Edited since 1922

POLISH GYNECOLOGY

# GINEKOLOGIA POLSKA

no 6/vol 91/2020

ORGAN POLSKIEGO TOWARZYSTWA GINEKOLOGÓW I POŁOŻNIKÓW
THE OFFICIAL JOURNAL OF THE POLISH SOCIETY OF GYNECOLOGISTS AND OBSTETRICIANS

IF: **0.941,** MNiSW: **40**

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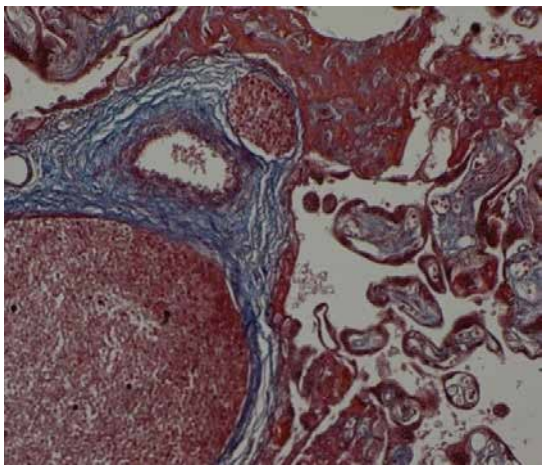

ISSN 0017–0011

VIA MEDICA

# PERINATOLOGIA I GINEKOLOGIA PRZEDŚWIĄTECZNIE

## Warszawa

11–12 grudnia 2020 roku

**Przewodniczący Komitetu Naukowego:**
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19-0431.001.011

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**Ginekologia Polska** is published monthly, twelve volumes a year, by VM Media sp. z o.o. VM Group sp.k.,
73 Świętokrzyska St, 80–180 Gdańsk, Poland, phone: (+48 58) 320 94 94, fax: (+48 58) 320 94 60,
e-mail: redakcja@viamedica.pl, marketing@viamedica.pl, http://www.viamedica.pl

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**Indexed in:** CrossRef, DOAJ, Index Copernicus, Ministry of Science and Higher Education (40), POL-Index, Polish Medical Bibliography, PubMed, Science Citation Index Expanded (0.941), Scimago Journal Rank, Scopus, Ulrich's Periodicals Directory

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19-0202.006.001

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## CONTENTS

## REVIEW PAPER
## GYNECOLOGY



## REVIEW PAPER
## OBSTETRICS



## RECOMMENDATIONS

VIA MEDICA

ORIGINAL PAPER / GYNECOLOGY

Ginekologia Polska
2020, vol. 91, no. 6, 295–300

ISSN 0017–0011

DOI 10.5603/GP.2020.0053

# The pain symptoms and mass recurrence rates after ovarian cystectomy or uni/bilateral oophorectomy procedures in patients over 40 years old with endometriosis

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## ABSTRACT

**Objectives:** To evaluate the rates of pain and mass recurrence of the patients over 40 years old with endometriosis who underwent ovarian cystectomy or uni/bilateral oophorectomy.

**Material and methods:** A retrospective study was conducted with 98 patients undergoing laparoscopic surgery for endometriosis in a tertiary referral center between the time period July 2015 and July 2019. All the patients followed every 3 months and requested to fill the Visual Analogue Scale (VAS) for evaluation of pelvic pain and an ultrasound scan was performed. The inclusion criteria for this study were as follows, patients with ages over 40, with regular menstrual periods, and who denied hysterectomy and any postoperative hormonal medical treatments.

**Results:** When the groups were compared in terms of age, body mass index, cyst diameter, CA-125 serum concentrations, preoperative and after surgical pelvic pain scores, mean follow up periods, postoperative hospital stay. However, each of the mean numbers of gravidity and parity were significantly higher than bilateral salpingo-oophorectomy (BSO) groups compared to the other groups ($p = 0.04$ and $p = 0.03$, respectively). The laterality, the recurrence rates, and the type of recurrence did not have a significant effect in the group comparison.

**Conclusions:** The ovarian tissue preserving procedures could be offered for the women over 40 years old suffering from endometriosis with no significant increase in pain symptom or mass recurrence rates considering beneficial effects of estrogen on cardiovascular system, vasomotor symptoms, and bone mineral density.

**Key words:** endometriosis; pain symptom; recurrence; oophorectomy

Ginekologia Polska 2020; 91, 6: 295–300

## INTRODUCTION

Endometriosis is characterized by the existence of endometrial-like tissue outside of the uterine cavity and especially endometriosis causes pelvic pain and infertility during oestrogen-dependent reproductive years. The most affected organ is the ovary, and this affected ovarian tissue is called a chocolate cyst or ovarian endometrioma. Ovarian endometriomas are observed in 17–44% of women with endometriosis [1].

Laparoscopic conservative surgery is recommended as the main surgical treatment in ovarian endometrioma [2–3]. However, the recurrence of endometrioma after surgery is a major problem. The rate of recurrence is very highly variable and reported with a range between 19% and 50% [4]. This recurrence may cause the reappearance of pelvic pain and requiring repeat surgery. The mechanism of recurrence is not fully understood, though it is generally believed to be as follows: the lesion is not removed completely, leading to recurrence from the residual lesion; eutopic endometrial tissue is the key factor in the onset of endometriosis according to the known retrograde menstruation theory [5]. In addition, there is no general agreement in evaluating the conditions that may cause the recurrence of endometrioma.

The age of the patient at the time of the operation was thought to affect the recurrence of endometriosis [6]. Until now, there has been a remarkable inadequacy of attention in the management opportunities for endometriosis patients who are past their reproductive years and in the natural transition towards menopause. However, compared with younger women, women older than 40 years may have more

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dilemmas during management, such as whether to retain ovarian tissue and whether it is necessary to add medical treatment after surgery in those for whom the use of oral contraceptives is no longer recommended [7]. Additionally, new studies of the factors related to postoperative recurrence in women over 40 years of age with endometriosis would help to provide references for the surgical strategy.

In conclusion, our study was aimed to determine the recurrence conditions after cystectomy or uni/bilateral oophorectomy surgeries for ovarian endometrioma in patients aged older than 40 years.

## MATERIAL AND METHODS

All premenopausal women over 40 years of age who experienced laparoscopic surgery for endometrioma were observed at our tertiary referral centre between January 2015 and January 2018.

The inclusion criteria were as follows: there was postoperative histopathological confirmation, patients had regular menstrual periods and denied the hysterectomy procedure and postoperative hormonal treatment, clinical follow-up and pathological data were completed, and patients were classified with stage 3/4 endometriosis patients according to the American Society of Reproductive Medicine (ASRM) classification [8]. The exclusion criteria were hysterectomy; ovarian endometrioma treated with ablation, drainage or incompletely stripping; postmenopausal status; other types of postoperative treatment, such as combined oral contraceptives, progestins, gonadotropin-releasing hormone agonists (GnRHa) or levonorgestrel-releasing intrauterine devices; and a histopathology of malignancy or borderline tumours.

The following data, including age, number of pregnancies, ovarian size (mm), laterality of ovarian cysts, preoperative and postoperative 3$^{rd}$ month visual analogue scales (VAS), body mass indexes, preoperative CA-125 levels, types of laparoscopic surgery, and recurrence during follow up (month) were retrospectively evaluated. Women were each requested for observation three months of the initial year and then annually.

The definition of recurrence of endometrioma was a radiologic appearance of an endometrioma with a circular-form cyst structure (at least 2 cm in size), surrounded with thick membrane, regularly boarded, and homogenous depleted echogenic substance with dispersed internal echogenic appearance, the absence of papillary structures, and/or pelvic pain [9].

In our study, we performed cystectomy, unilateral salpingo-oophorectomy (USO) and bilateral salpingo-oophorectomy (BSO), and the data from these three groups of patients were analysed.

The surgery was done throughout an umbilical insertion and right and left infra- umbilical insertions by 5 mm grasping forceps and scissors. Pneumoperitoneum was obtained using a Veress needle (Ethicon Endo-surgery Inc., USA) insertion. In all cases $CO_2$ was used with 12 mmHg intrabdominal pressure. An incision was performed on the endometrioma, and the tissues were dissected, and then exposed. After aspiration of the cyst contents, pathological tissue was pulled from different angles and separated from ovarian parenchyma. Haemostasis was accomplished by bipolar coagulation. Additionally, laparoscopic USO and BSO procedures were performed with the same process and subsequent infundibulopelvic ligament identification and adnexal removal. When the adhesion was observed endometrioma were released from normal tissue by different cleavages. After endometriomas were removed visible endometriotic lesions were excised or coagulated with bipolar energy. Anatomical restoration was then achieved in all cases.

Data analysis was achieved with SPSS (version 20.0; SPSS Inc., Chicago, IL, USA). All data were shown as mean ± standard deviation. A one-sample Kolmogorov–Smirnov test was accomplished to examine the dispersion of clinical and surgical outcomes. Parametric variables were analyzed by Student's t-test; non-parametric variables were analyzed by Mann–Whitney U test and chi-square test. A p-value of $< 0.05$ was determined to be statistically significant for analysis.

## RESULTS

A total of 98 patients who had an adequate after surgical follow-up and met the inclusion criteria were participated in the study. The main groups of study were cystectomy patients (n = 30), USO patients (n = 39) and BSO patients (n = 29). The baseline characteristics are shown in Table 1. When the groups were compared, there was no significant effect of patient age during the operation. However, the mean numbers of both gravidity and parity were significantly higher in the BSO group compared to the other groups ($p = 0.04$ and $p = 0.03$ respectively). There were no significant differences between the groups regarding body mass index, preoperative CA-125 levels, preoperative and postoperative third month VAS scores, the number of previous endometriosis surgeries, ovarian lesion size or postoperative hospital stay. The mean postoperative follow-up was 23.0 months (range 3–34 months) in all women. There were also no significant differences in the recurrence time after surgery between the groups.

The risk factors associated with the endometrioma recurrence are presented in Table 2. There were no differences between the groups regarding the laterality of lesions or the usage of preoperative medication. Additionally, the presence of a pouch of Douglas obliteration did not significantly affect the recurrence rate.

**Table 1.** Comparison of the basic characteristics of the study groups

| | Cystectomy (n: 30) | USO (n: 39) | BSO (n: 29) | p value |
|---|---|---|---|---|
| Age [years] | 41.86 ± 3.27 | 42.64 ± 3.08 | 43.34 ± 3.16 | 0.1 |
| Gravidity | 1.36 ± 1.15 | 1.58 ± 0.71 | 1.93 ± 1.09[a,b,c] | **0.04** |
| Parity | 1.31 ± 1.11 | 1.55 ± 0.69 | 1.82 ± 1.1[d,e,f] | **0.03** |
| BMI [kg/m$^2$] | 24.14 ± 2.35 | 25.89 ± 3.57 | 26.23 ± 4.27 | 0.07 |
| Size [mm] | 70.3 ± 34.83 | 66.43 ± 26.33 | 53.85 ± 23.07 | 0.27 |
| CA-125 IU/mL | 85.43 ± 129.32 | 96.49 ± 181.64 | 73.36 ± 68.03 | 0.66 |
| Pre-op Pelvic pain [VAS] | 8 (0–10) | 8 (0–10) | 8 (0–10) | 0.99 |
| Post-op 3$^{rd}$ month Pelvic pain [VAS] | 3.5 (0–7) | 4 (0–8) | 3 (0–9) | 0.61 |
| Number of previous endometriosis surgeries | 0.16 ± 0.46 | 0.23 ± 0.58 | 0.13 ± 0.35 | 0.83 |
| Mean follow-up [months] | 24.26 ± 10.49 | 22.10 ± 10.75 | 22.51 ± 9.77 | 0.51 |
| Hospital stay [days] | 3.63 ± 1.71 | 3.23 ± 1.11 | 3.96 ± 2 | 0.40 |
| Recurrence time [months] | 2.83 ± 7.79 | 2.61 ± 8.11 | 0.93 ± 4.47 | 0.69 |

A p value of < 0.05 was considered significant for all bold values; USO — unilateral salpingo-oophorectomy; BSO — bilateral salpingo-oophorectomy; VAS — visual analogue scale; BMI — body mass index; [a, d] — p < 0.001 (cystectomy vs USO), [b, e] — p < 0.001 (cystectomy vs BSO), [c, f] — p < 0.001 (USO vs BSO)

**Table 2.** Comparison of the risk factors associated with the endometrioma recurrence of the study groups

| | | Cystectomy | USO | BSO | p value |
|---|---|---|---|---|---|
| Laterality | Unilateral | 21 (70%) | 24 (61.5%) | 13 (44.8%) | 0.06 |
| | Bilateral | 9 (30%) | 15 (38.5%) | 16 (55.2%) | |
| Preoperative medication | Yes | 8 (26.7%) | 11 (28.2%) | 7 (24.1%) | 0.82 |
| | No | 22 (73.3%) | 28 (71.8%) | 22 (75.9%) | |
| Douglas obliteration | Yes | 22 (73.3%) | 28 (71.8%) | 18 (62.1%) | 0.35 |
| | No | 8 (26.2%) | 11 (28.2%) | 11(37.9%) | |
| Recurrence rate | Yes | 4 (13.3%) | 4 (10.3%) | 2 (6.9%) | 0.41 |
| | No | 26 (86.7%) | 35 (89.7%) | 27 (93.1%) | |
| Type of recurrence | Pain | 2(6.7%) | 3(7.7%) | 2(6.9%) | 0.58 |
| | Mass | 2 (6.7%) | 1(2.6%) | 0 | |
| | None | 26 (86.7%) | 35 (89.7%) | 27 (93.1%) | |

USO — unilateral salpingo-oophorectomy; BSO — bilateral salpingo-oophorectomy; a p value of < 0.05 was considered significant for all bold values

The recurrence rate following endometrioma surgery was 13.3% in the cystectomy group, 10.3% in the USO group and 6.9% in the BSO group. There were no statistically significant differences in the recurrence rate between the groups. Additionally, a type of recurrence analysis was conducted between the cystectomy group (pain 6.7%, mass 6.7%), USO group (pain 7.7%, mass 2.6%), and BSO group (pain 6.9%, mass 0%); and there were no statistically significant difference within these groups.

## DISCUSSION

This study evaluated the rates of pain and mass recurrence in patients over 40 years old with endometriosis who underwent ovarian cystectomy or uni/bilateral oophorectomy surgeries. The recurrence rate reported in the literature varies significantly from 6% to 67% due to different follow-up durations, different surgical techniques and different diagnostic criteria [11–12]. Age-dependent endometriosis data have demonstrated that a younger age during the endometriosis operation is related with recurrence of endometrioma [13–14]. Another study showed that endometrioma recurrence was higher in patients undergoing an endometriosis operation under the age of 32 [15]. In younger women, endometriomas may be more aggressive due to a high level of hormones, consequently it may cause more recurrence rates [15–16]. Additionally, a study showed that endometriosis might develop from follicles or the corpus luteum [17]. On the other hand, this issue was discussed for the first time in 1993 by Witt and Barad with a study proposed at discussing the treatment modalities in women with an endometrioma above 40 years of age [18].

Oestrogen plays important roles in processes that protect heart and bone physiology [19]. Along with these significant effects of oestrogen, the physical changes during the perimenopausal years are established in hormonal alterations, particularly variations in the level of circulating oestrogen. This premenopausal population faces extraordinary problems while managing the effects of endometriosis on the patient. Some of these comprehended the elevated risk of medical contraindications to combined hormonal contraceptives (COC) and prolonged disease significantly increases the risk of cancer, with the possibility of causing malignant transformation [20]. Because of this condition, and considering the different types of laparoscopic surgeries, the management of oestrogen-dependent diseases such as endometriosis may become complicated in women over 40 years old due to the severe alterations of oestrogen levels. According to these data, our study is focused on determining the rate of recurrence in patients over 40 years old with endometriosis who underwent different type of laparoscopic surgeries.

Variations in recommended recurrence criteria, follow-up time, and study populations may result in different rates of recurrence rates of endometrioma in the literature [21]. Chan et al. [22] founded that dysmenorrhea and septated ovarian cysts significantly altered the recurrence rate after surgery. Additionally, a study showed that the depth of invasion of the endometrial tissue into the normal tissue was an independent risk factor for recurrence rate of endometrioma [23]. Another study showed that CA-125 serum concentrations, cyst diameter and previous pelvic surgery would affect the recurrence rate [24]. Our study showed no significant association between recurrence rate and patient-related factors such as gravidity, parity, preoperative serum CA-125 level, body mass index and cyst diameter. Additionally, Campo et al. [25] investigated that the relationship between endometrioma recurrence and surgical situations such as mass size, number and laterality, adhesions or implantations, existence of spillage and demographic situations such as age, body mass index, family history; the authors showed that family history of endometriosis was the only factor affecting endometrioma recurrence rate. On the other hand, another study suggested that the cumulative recurrence rate of ovarian endometrioma was related with the since onset of time follow-up and severe endometriosis, as the second-line surgery seemed to be a risk factor associated with the increased recurrence rate [26]. Previous studies concluded that the cumulative recurrence rates of conservative surgery, semi-radical surgery and radical surgery were 28.9%, 17.3% and 0% respectively [27]. In our study, the cumulative recurrence rates in the cystectomy, USO and BSO surgery group were 13.3%, 10.3% and 6.9%, respectively.

The international guidelines on endometrioma have not recommended exact suggestions for the management of patient approaching menopause, likely due to the lack of evidence. The 2014 European Society of Human Reproduction and Embryology guidelines suggest considering definitive operation (hysterectomy with bilateral oophorectomy, together with the removal of other lesions) for women who terminate their reproductive life and had persisting symptoms despite medical therapy [28]. The guidelines also proposed to brief the women in whom hysterectomy would not necessarily treat endometriosis symptoms. Alike recommendations have been suggested by the Society of Obstetricians and Gynecology of Canada [29]. In addition, the World Endometriosis Society consensus on the present treatment of endometriosis has announced that suggestions were associated to women of reproductive age but emphasized that the role of hysterectomy and simultaneous oophorectomy demonstrates the ensuring a little reliable evidence effect [30]. On the other hand, in recent years, an increasing number of patients have expected to preserve the ovaries and the uterus. All patients included in our study denied hysterectomy procedure. Additionally, clinicians pay more attention to the preservation of reproductive organs in perimenopausal patients due to the beneficial effects of oestrogen levels. Furthermore, additional operations may increase the duration of surgery and morbidity. Zing et al. [7] showed that the ovarian preservation surgery was an independent risk factor for recurrence in patients with ovarian endometriosis (> 45 year old). However, our results showed no significant difference in recurrence rate with different types of endometrioma surgeries.

In different studies evaluating the pathophysiology of recurrence, it has been suggested that the recurrent lesion may derived from remaining tissue. Failure to completely remove pathological tissues at the time of primary surgery may increase the likelihood of recurrence to endometrioma, and the larger part of recurrent lesions were formed in the same area [31–32]. Thus, during the primary surgery for endometriosis, an excellent surgical technique and the experienced endometrioma surgeons are critical due to the capability of pathologic residual tissue to grow.

For many years, the medications used in the management of endometriosis included selective oestrogen receptor modulators, aromatase inhibitors, GnRHa, selective progesterone receptor modulators and angiogenic agents. A Cochrane review on the use of postoperative medication in endometriosis suggested that there was no evidence of any improved pain relief, though the level of evidence was not sufficient in the reported literature [5]. However, A study found that use of GnRHa after surgery could only extend the recurrence interval but not decrease the whole recurrence rate [33]. In our study, endometrioma

management included only surgery without the addition of postoperative medications.

However, our study also has a few limitations. First the study was not a randomized controlled study; therefore, some possible biases could have occurred. In addition, our study focused on different types of adnexal surgeries, and hysterectomy operations could be added for the analysis. Additionally, patients with histopathology of malignancy or borderline tumours were excluded from the study due to the requirement of oncologic management. This situation may cause additional limitation in our study. Although endometriosis-associated malignant ovarian tumours are usually early stage, oncologic safety of the surgical procedures such as cystectomy could not be analyzed due to this limitation [34]. Finally, this study had a relatively small sample size.

In conclusion, the rate and prevention of disease recurrence in women approaching menopause are unknown factors. Our study showed that ovarian tissue-preserving procedures could be offered for the women over 40 years old suffering from endometriosis with no significant increase in pain symptoms or mass recurrence rates considering the beneficial effect of oestrogen on the cardiovascular system, vasomotor symptoms and bone mineral density.

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VIA MEDICA

ORIGINAL PAPER / GYNECOLOGY

Ginekologia Polska
2020, vol. 91, no. 6, 301–307

ISSN 0017–0011

DOI 10.5603/GP.2020.0055

# A preliminary study on the immune responses of HPV16-E7 by combined intranasal immunization with lymphotoxin

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## ABSTRACT

**Objectives:** Human papillomavirus (HPV) ranks the first cause of cervical cancer. Cervical cancer has high prevalence rates in women around the world. The HPV-E7 oncoprotein is expressed in cervical cancer and is a target of developing immunotherapies against HPV-associated tumors. However, the antigenicity of this protein is low. Due to this reason, potent adjuvants are required to enhance its therapeutic efficacy. This preliminary study aims to evaluate whether lymphotoxin (LT) could act as an effective immune adjuvant for HPV infection in mice models.

**Material and methods:** Intranasal immunization was used to explore the effect of HPV-E7 and/or LT immune response. After the third intranasal immunization, the titer for the HPV-E7 antibody was detected in serum and vaginal washing fluid. Also, we assessed the expression of chemokine ligand 13 (CXCL13) and Peripheral Node Addressin (PNAd) in the lymph nodes after intranasal immunization with immunohistochemical analysis.

**Results:** compared to HPV-E7 immunization, intranasal immunization with HPV-E7 plus LT significantly increased HPV-E7-specific serum IgG and vaginal IgA titers. Furthermore, the combined use of HPV-E7 and LT strongly induced E7-specific CTL responses.

**Conclusions:** LT can be effective for intranasal immunized HPV-E7 to improve E7-specific immune responses to HPV infection. It is new approach to eradicate chronic HPV infection capable of inducing an effective anti-infection method.

**Key words:** human papillomavirus; HPV-E7; lymphotoxin; intranasal immunization; immunotherapy; lymphocyte homing

Ginekologia Polska 2020; 91, 6: 301–307

## INTRODUCTION

Cervical cancer has high prevalence rates in women around the world [1, 2]. According to the World Health Organization (WHO) Human Papillomavirus and Related Cancers, World Summary Report 2010, every year, worldwide, HPV caused 500,000 new cases of cervical cancer [2]. Most HPV infections are harmless and clear up spontaneously; however, persistent HPV infections (especially type 16) have been reported could cause many human cancers such as cervical cancer and oropharyngeal cancer [3, 4].

HPV generally encodes six non-structural proteins (E1, E2, E6, E7 E5, E4) and two structural proteins (L1 and L2). These HPV encoded antigens are expressed differentially across the maturing cervical epithelium [1]. It has been reported that natural immune responses to HPV encoded antigens are typically weak and the immune responses vary among individuals, but the E7 protein is an exception to this [5]. The E7 protein is one of the proteins that could elicit a humoral immune response in most cases of invasive cervical cancer [5]. Due to its interactions with regulatory cellular proteins, *e.g.*, p53 and pRb, HPV E7 protein could disrupt the normal cell cycle and the process of DNA repair, leading to neoplastic transformation and the immortalization of epithelial cervical cells. Therefore, E7 is regarded as a powerful transforming oncoprotein target protein for HPV therapeutic strategies. It can directly against HPV infection to prevent and treat the cervical neoplasia [6].

There have been many different attempts to induce E7 specific immune responses in previous studies [7]. For example, Yang et al. [8] conducted a study and found that E7 peptide could induce antigen-specific T cells in animal subjects. However, to date, researchers have not been able to clinically demonstrate an effective therapeutic approach using this method. Several likely reasons exist for this, in-

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cluding the ability of HPV to evade immune recognition [9]. Therefore, there is an urgent need for novel therapies in order to eradicate HPV infection in the genital tract mucous membrane.

Lymphotoxin (LT) is one of the members in tumor necrosis factor (TNF) superfamily that has been shown to be an important component of effector immune responses. LT receptor pathway is essential to protect innate and adaptive immune and LT is essential for host defenses against specific pathogens, for example, it's involved in the production of IgA and many other cytokines [10]. Lymphotoxin (LT) plays multiple roles in the immune system and autoimmunity. It's reported that mucosal immunity is more effective against clear pathogens of mucous membrane infection than traditional immunization like oral administration and injection immunization [7]. Mucosal immunization can be achieved via oral, intranasal, pulmonary, rectal, or vaginal routes, and intranasal immunization with purified HPV16 VLPs induces systemic and mucosal antibody responses [7].Compared to vaginal and rectal mucosal immunity, intranasal immunization is an ideal method to induce genital mucosal immune response because it is cost-effective and convenient [11, 12].

In consideration of the above, we conducted this preliminary study to explore whether co-administration of E7 peptides and LT with the addition of cholera toxin (CT) elicits immune responses as well as protects against the tumor. Our results showed that intranasal co-administration of E7 peptides and LT induced stronger antigen-specific immune responses in mice when compared to the response from E7 alone. Further, LT stimulation significantly enhanced E7-specific T-cell responses and CTL responses. We also demonstrated that co-administration of E7 peptides with LT could induce lymphocyte homing in the mouse models.

## MATERIAL AND METHODS

### Animals

All C57BL/6 mice (female, 6–8 weeks old) were purchased from Animal Center of Nanjing Medical University (Nanjing, China). Animals in this study were kept in the pathogen-free conditions. All animal care and experimental procedures were performed in accordance with recommendations for the proper use and care of laboratory animals.

The study was approved by the Ethics Committee of our hospital.

### Recombinant E7 peptide and LT

Recombinant peptide of HPV-16 E7 protein (amino acids: 31–50 SSEEEDEIDGPAGQ AEPDRA) was synthesized by the GL Biochem (Shanghai, China). The peptide purity was above 98% as determined by reverse-phase high-performance liquid chromatography. E7 peptide was dissolved in double-distilled $H_2O$. All peptides that were not immediately used were stored at –20° C. LT with a purity of > 98%. was purchased from PeproTech (NJ, USA).

### Immunization of mice

Twenty-four mice were randomly assigned to four groups (n = 6 each): PBS control group, LT group, HPV-E7 group and HPV-E7 plus LT group. The mice in the four groups were lightly anesthetized with 10% ketamine and 5% xylazine in sterile water for the duration of the inoculation. The mice in HPV-E7 + LT group were immunized intranasally with 10 µg of HPV-E7 and 2 µg of cholera toxin (Sigma- Aldrich, MO, USA) and injected through the vena caudalis with 0.05 µg of Lymphotoxin in a total volume of 20 µL per mouse. The mice in HPV-E7 group were immunized intranasally with 10 µg of HPV-E7 and 2 µg of cholera toxin alone. The mice in the LT group received 0.05 µg of Lymphotoxin in a total volume of 20 µL per mouse and the PBS control group received PBS only. All mice in the four groups were immunized three times with the same dosage of the corresponding immune formulation at two-week intervals. The mice in control group were given PBS in the same volume on days 0, 14, and 28.

### Collection of vaginal samples and serum

Samples of blood and vaginal were collected on day 0, 14, 28, and 42 following intranasal immunization and/or LT injection. Blood samples were obtained by retro-orbital sinus puncture and clotted at room temperature for 60 minutes. After microcentrifuged at 1,700 g for 10 minutes, the serum was separated and then stored immediately at –20° C until analysis. In order to obtain the vaginal samples, 100 µL PBS were injected into the vaginal cavity. The vaginal samples were then washed five times in the lab.

### Antibody Detection

HPV-E7 antibody titers were detected using enzyme-linked immunosorbent assay at dilutions of 100, 200, 400, 800, 1600, 3200 for detections of IgG, and at delusions of 10, 20, 40 for detections of IgA. For detection of HPV-E7-specific vaginal IgA and serum IgG, high protein-binding 96-well plates (Invitrogen, Burlington, ON, Canada) were incubated overnight at 4°C, with a capture layer of 100 µL HPV-E7 peptide (50 µg/mL in carbonate buffer, pH 9.6). After three washes with PBS-0.05% Tween-20, the plates were blocked with 3% bovine serum albumin in PBS at room temperature for 2 to 4 hours, then samples were applied in duplicate at various dilutions in 3% bovine serum albumin-PBS as indicated in individual experiments, and incubated at 4° C overnight. The plates were washed five times, incubated with peroxidase-conjugated goat anti-mouse IgA (Santa Cruz, CA, USA), diluted 1:5000 in 1% bovine serum albumin-PBS or anti-mouse IgG (Santa Cruz, CA, USA), then diluted 1:2500, at room temperature for 1 hour.

After seven washes, 100 µL of TMB (Sigma Aldrich, MO, USA) were added to each well. Colorimetric reactions were performed at room temperature in the dark then stopped by adding 1N $H_2SO_4$. ODs were measured at 450 nm using a 96-well spectrophotometer (Microplate Reader Model 680, BIO-RAD, USA). End point titers were determined and expressed as geometric mean titers. The mean background optical density value of non-immune mice was measured twice and used as the cutoff for determining positive values.

### DC preparation

Spleen DC was obtained aseptically from C57BL/6 mice and placed in the PBS buffer. After dissociation, suspension was collected through a 400-mesh steel net. First, red blood cells were removed by adding ACK solution (Biofluids, Camarillo, CA) and the remaining cells were resuspended with RPMI1640 medium containing 10% fetal bovine serum, then counted. Non-adherent cells were disposed of and the remaining cells were divided into two parts. Spleen cells were labeled with 2.5 µmol/L Carboxy Fluoroscein Succinimidyl Ester (5- or 6-(N- uccinimidyloxycarbonyl)-3′,6′-0,0′-diacetyl-fluorescein, CFSE; Invitrogen, USA) (CFSE high) or 0.25 µmol/L CFSE (CFSE low). CFSE high cells were then pulsed for 90 min at 37°C with either 2 µg/mL HPV-E7 or a survivin peptide as a control in a 5% $CO_2$ incubator. After extensive washing, CFSE-high and low cells were mixed in a 1:1 ratio.

### Flow cytometric analysis

After being stimulated three times, the CFSE-high and CFSF-low cells ($2 \times 10^6$, 100 µL) were mixed in a ratio of 1:1 and injected intravenously or vaginally under light anesthesia. After 12 hours of administration, the spleen and uterus were processed into single cell suspensions. Briefly, after perfusion with cold 1x PBS, the spleen was removed, minced into fragments, and digested with 1 mg/mL collagenase (catalog no. 17018–029; Gibco) and 0.1 mg/mL DNAase for 1 hour at 37°C with intermittent agitation. Spleen fragments were passed through a 400-mesh steel net (Falcon; BD Biosciences). and approximately 13–107 cells were incubated in 2.5 mg/mL Fc blocking solution, centrifuged (800 × g, 10 minutes, 8°C), and resuspended with FACS buffer.

Approximately $1 \times 10^5$ to $10^6$ cells per sample were measured to assess the clearance rate of the target cells. The following formula was used: the specific killing rate (%) = [1 – (CFSE-low control/CFSE-high control)/(CFSE-low experimental/CFSE-high experimental)] × 100%. The formula was used to measure the percent of cells that were killed. Data were acquired on a Becton Dickinson FACSCalibur flow cytometer and analyzed with FlowJo software (Tree Star Inc., Ashland, OR, USA).

### Statistical analysis

All data are expressed as mean ± SD. Graphpad Prism (version 5; San Diego, USA) was used to construct figures and diagrams. One-way analysis of variance (ANOVA) or unpaired T-tests and Mann–Whitney tests were used where appropriate. Differences were considered statistically significant if the p-value was less than 0.05. Error bars represent ± SD.

## RESULTS

### HPV-E7 plus LT enhances E7-specific systemic humoral immune response

To evaluate the adjuvant properties of LT *in vivo*, the geometric mean titers of the antigen-specific antibody were analyzed in this study. At two-week intervals, mice were administrated three times with the indicated amount of HPV-E7 plus LT, HPV-E7, LT, or PBS. In addition, CT was added in each group as a mucosal adjuvant. For 42 days, serum samples were collected after intranasal immunization and titrated for HPV-E7-specific antibodies. Animals immunized with HPV-E7 showed detectable HPV-E7-specific antibodies. HPV-E7 plus LT elicited a significantly higher increase of HPV-E7-specific antibody levels compared to inoculation with HPV-E7 alone. However, the mice inoculated with LT alone did not show any increase in humoral immune response (Fig. 1). These results indicated that LT could significantly enhance the HPV-E7 peptide-specific humoral immune response.

### HPV-E7 plus LT enhances E7-specific vaginal humoral immune response

We also examined the adjuvant properties of LT in the HPV-E7-specific vaginal humoral immune response. The level of vaginal IgA was 1:40 in the HPV-E7 plus LT group and 1:20 in the HPV-E7 group (Fig. 2). At the dilution level of 1:20 and 1:40, the HPV-E7-specific antibody titers were higher in the HPV-E7 plus LT group than those in the HPV-E7 group. Our results demonstrated that a significant

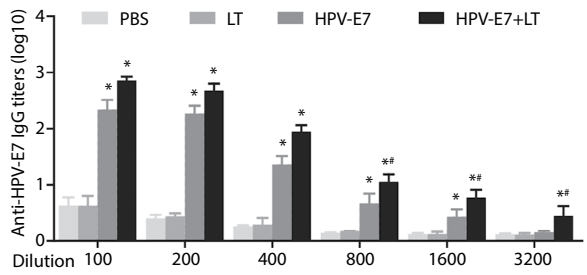


**Figure 1.** Antibody response to HPV-E7 and/or LT *in vivo*. Mice were immunized with PBS, HPV-E7 and/or LT at two-week intervals. Serum was collected 42 days after immunization and diluted into different concentration. IgG responses against HPV-E7 were evaluated using ELISA; *$p < 0.05$ as compared to PBS control mice; #$p < 0.05$ as compared to HPV-E7 naive mice

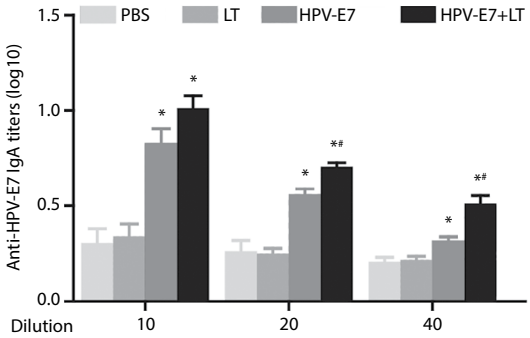


**Figure 2.** HPV E7-specific vaginal humoral immune response to HPV-E7 and/or LT *in vivo*. C57BL/6 mice were immunized with HPV-E7 and/or LT at two-week intervals. Vaginal washes were collected after HPV-E7 and/or LT immunization for 42 days and diluted into different concentrations. HPV-E7-specific IgA antibodies were evaluated using ELISA; *p < 0.05 as compared to PBS control mice; #p < 0.05 as compared to HPV-E7 naive mice

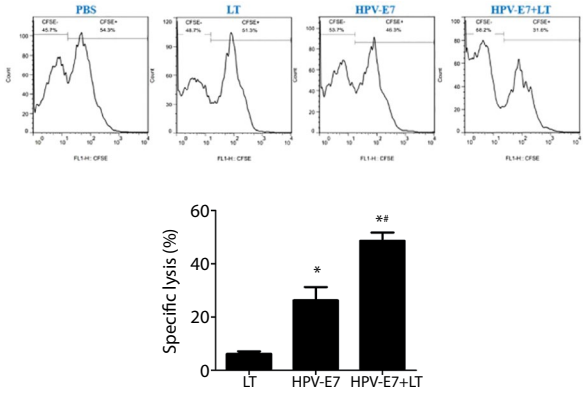


**Figure 3.** Mice were immunized with PBS, HPV-E7 and/or LT at two-week intervals then injected with syngeneic CFSE-labeled splenocytes pulsed with E7 peptide to measure spontaneous cytotoxicity generated by CTL cells. The peptide-specific killing response *in vivo* was determined 7 d later. The killing rate of CTL was calculated using the following formula: the specific killing rate (%) = [1 – (CFSE-low control/CFSE-high control)/(CFSE-low experimental/CFSE-high experimental)] × 100%; *p < 0.05 as compared to PBS control mice; #p < 0.05 as compared to HPV-E7 naive mice

HPV-E7-specific vaginal humoral immune response was induced by LT.

## Immunization with HPV-E7 peptide plus LT stimulated enhanced systemic anti-HPV-E7 CTL responses

We investigated whether HPV-E7 + LT immunization was able to induce anti-HPV-E7 CTL responses. The mice were immunized three times with HPV-E7 + LT, HPV-E7or PBS. The splenocytes of the immunized mice were stimulated with HPV-E7 and cytometric analysis was used to detect the activities of the target cells killed by HPV-E7-specific CTL. M1 represented CFSF-low cells and M2 represented

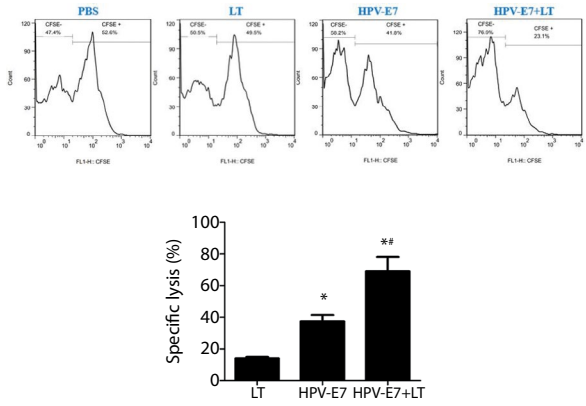


**Figure 4.** Mice were immunized with PBS, HPV-E7, and/or LT then injected with syngeneic CFSE-labeled splenocytes pulsed with E7 peptide to measure vaginal specific cytotoxicity generated by CTL cells. HPV-E7-specific killing response in vaginal washes was determined 7 d later; *p < 0.05 as compared to PBS control mice; #p < 0.05 as compared to HPV-E7 naive mice

CFSE-high cells (Fig. 3). M1 and M2 cells in the control group were substantially equal, there were no obvious cytotoxicities of CTL or killing effects for target cells incubated with HPV-E7. There were fewer M2 cells than M1 cells in both the HPV-E7 and HPV-E7 + LT groups. This means that immunization with HPV-E7 plus LT induced a significant increase of the HPV-E7-specific killing response *in vivo*. The killing rate of HPV-E7 + LT group was higher than that in HPV-E7 alone group (51.64% ± 3.07% vs 26.25% ± 5.04%, p < 0.05). And the killing rate of the PBS group was (6.16% ± 0.97%).

## HPV-E7 peptide plus LT stimulated enhanced E7-specific vaginal CTL responses

To determine whether LT modulates a vaginal HPV-E7-specific response, vaginal samples were obtained and HPV-E7-specific IgA were examined. We found that the killing rates of the HPV-E7 + LT group increased significantly compared to HPV-E7 immunization alone (69.12% ± 9.01% vs 37.46 ± 4.03%, p < 0.05). Our results suggested that LT played an important role in the effect of CTL-mediated cytotoxicities (Fig. 4).

## HPV-E7 peptide plus LT induces lymph homing

In the present study, we assessed the capacity of protective T-cell subpopulations to home to the genital mucosa and associated lymphoid tissues in response to HPV-E7 and LT immunization. Tissue section morphometry of lymph nodes showed the mice that were immunized with HPV-E7 + LT had the most abundant CXCL13 and PNAd levels. The mice that were immunized with either PBS or LT alone had the lowest CXCL13 and PNAd levels. There were no differences in the PBS and LT immunization positive

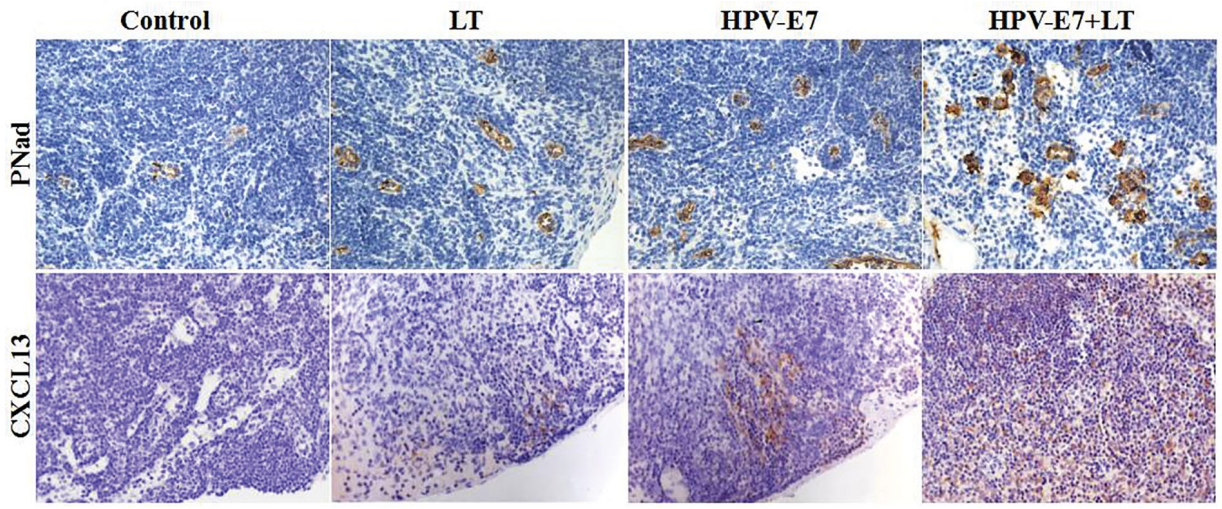


**Figure 5.** Mice were immunized with PBS, HPV-E7 and/or LT at two-week intervals. Representative images for the immunohistochemical staining of PNAd and CXCL13 in lymph nodes of different groups (400×)

areas regarding CXCL13 and PNAd. These results demonstrate that HPV-E7 immunization contributes to lymphocyte homing to genital mucosa. Our findings that lymph nodes with HPV-E7+LT immunization express stronger lymphocyte "homing" chemokines CXCL13 and PNAd than those immunized with HPV-E7 alone, suggest that LT is critical to promoting lymphocyte homing induced by HPV-E7 immunization (Fig. 5).

## DISCUSSION

Each year, worldwide, there are approximately 529,000 newly diagnosed cases of cervical cancer, which have approximately a 50% mortality rate [13]. In the occurrence of cervical cancer, HPV plays an important role. Traditional methods to manage cervical cancer are focused on resolving the tumor. The methods to treat viral infection are usually neglected. Therefore, recurrence of cervical cancer often happens after successful treatment. To effectively eliminate HPV precancerous lesions and eliminate recurrence, therapeutic treatments for HPV infection are required. It has been reported that peptide-based immunotherapy is promising for resolving both cancer and HPV infection, but its full potential has yet to be investigated and its inherent limitations have not been overcome [14]. High-risk HPV-E7 is generally known as the most important oncogene of cervical cancer [6]. E7 is an HPV protein that is expressed during early phases of viral infections. Data indicates that E7 is capable of activating the specific CTL cells which act as effector cells to eliminate HPV infected cells by directly killing or releasing tumor inhibitive factors [15]. In this study we tested HPV-E7 as a target for an effective immune therapy and immune regulatory factors to eliminate HPV infection in the genital tract. It was difficult to induce intense immunoreactions and clear pathogens adhering to mucous surface using conventional subcutaneous immunization in genital mucosa. We chose to deliver HPV-E7 via the nasal route because this route had higher mucosal antibody titers than the other mucosal routes of immunization (oral, rectal, and vaginal) [15]. Also, we use CT to enhance HPV-E7-specific antibody response.

### Intranasal immunization with HPV-E7 peptide stimulated enhanced E7-specific systemic and vaginal humoral and cellular immune responses

Most HPV infections begin at the mucosal surfaces; therefore, immunity against HPV infection might depend on the mucosal immune response [16]. This makes the mucosal route the most appropriate way for immunization due to its ability to induce both mucosal and systemic immune responses [16]. Mucosal immunity is a special part of the immune system and is associated with most lymphoid tissues (> 50%) characterized by common mucosal effects, including gut, nasal and urogenital associated lymphoid tissues [17]. Plasma cells and sensitized lymphocytes activated in inductive sites home to diffuse lymphoid tissues of various mucosae through interaction with lymphocyte homing receptors, endothelial cell adhesion molecules, and corresponding peripheral node addressins to induce immune response. Humoral and cell-mediated antigen immune responses can be induced by mucosal immunization. Mucosal immunization can be achieved via oral, intranasal, pulmonary, rectal, or vaginal routes [18–19]. It has been reported that intranasal immunization with purified HPV16 VLPs induces systemic and mucosal antibody responses [20]. Therefore, we used intranasal immunization to explore the effect of HPV-E7 and/or LT immune response.

Also, CT was used as a mucosal adjuvant to enhance mucosal antibody response.

Two weeks after the third intranasal immunization in C57BL/6 mice, the titer for the HPV-E7 specific antibody was detected in the serum and vaginal washing fluid, following CT treatment. Our results showed that the titers for the HPV-E7 specific antibody in the serum and vaginal washing fluid were higher than in the control group following administration of CT, which suggested that appropriate humoral immunity responses were generated after HPV-E7 intranasal immunization.

LT is one of the members in the TNF superfamily and was originally identified as a lymphocyte product that was able to exert cytotoxic effects on tumor cells *in vitro* [10, 21]. Previous studies have found that LT-α and its complex play crucial roles in nasal-associated lymphoid tissue development and function [22]. The cytokine LT is a promising candidate for use in cancer therapy. It is able to kill various specific cancer cells. Therefore, LT has received increased public attention and is considered a promising antitumor and antivirus biological agent.

In our study, we showed that HPV-E7, with the help of an LT adjuvant, following CT treatment, can elicit a significant HPV-E7-specific response in the immune system and vaginal tract. We also found that HPV-E7 plus LT can elicit a strong E7-specific immune response *in vivo*, as evidenced by a significantly enhanced systemic and vaginal humoral immune response, cytotoxicity, and lymph homing, compared with control groups. The data presented here demonstrated that LT markedly promoted humoral and cellular responses of the immune system and genital tract mucosa after intranasal immunization with HPV-E7 following administration of CT. LT can effectively strengthen the clearance rate of HPV infected cells in the local genital tract by HPV-E7. It should be noted that the differences between HPV-E7 plus LT and HPV-E7 were only significant at high dilutions, which needs further explorations in future studies and also needs improved to achieve a goal that at a much lower dilution, HPV-E7 plus LT can demonstrate a significant effect.

Hydrogen-bonded multilayered Tannic Acid (TA), a novel nanothin polymer material, showed similar effects to LT in anticancer immunotherapy [23]. TA was designed through hydrogen-bonded interactions of a natural polyphenol with poly (N-vinylpyrrolidone) (PVPON) deposited via layer-by-layer (LbL) assembly. The combination of TA and HPV-E7 with LT will be considered in future research [24].

### LT stimulating HPV-E7 intranasal immunization by inducing lymph homing

The mucosal immune system is the first line to defense against pathogen invasion. Different parts of the mucosa can be connected by lymphocyte homing, generating a common immunological effect. The lymphocyte homing process is related to a variety of migrated molecule cascades that are involved in the lymphocyte homing process. These include lymphocyte homing receptors, addressins, and lymphatic chemotactic factors and their receptors. L-selectin, one of the lymphocyte homing receptors, is a member of the cellular adhesion molecular selectin family that participates in the process of lymphocyte homing and recycling [25]. PNAd is the ligand of L-selectin. It is first obtained from high endothelial venules of mice lymphocytes by monoclonal antibody MECA-79 and over-expressed in nasal-associated lymphoid tissue [26]. Lymphatic chemotactic factor CXCL13, also known as B-cell chemotactic factor, is a member of CXC chemotactic factor family. CXCL13 can induce the migration and homing of mature B lymphocytes after integrating with its receptors.

This preliminary study found that adhesion molecules, lymphatic chemotactic factors, and addressins were low-expressed or non-expressed in LT knockout mice [22]. In this study, we used the lymphocyte homing related factors CXCL13 and PNAd to explore the possible target factors adjusted by LT in the lymphocyte homing pathway after intranasal immunization with HPV-E7. We found that the expression of CXCL13 and PNAd was elevated significantly in lymph nodes with HPV-E7 + LT immunization compared to those immunized with HPV-E7 alone. This demonstrates that LT regulates the immunological effects of intranasal immunization by stimulating activation and homing of lymphocytes.

The immunogen used in this study is a recombinant peptide of HPV-16 E7 protein, which may not be able to protect against other subtypes of HPV virus. In addition, intranasal vaccination may not be so effective as intravaginal vaccination, but its advantage is that it's convenient and has lower risks of infections.

In conclusion, specific humoral and cellular immune responses of the immune system and genital tract can be induced by intranasal administration of HPV-E7. LT can effectively promote the effects of intranasal immunization via regulating expression of lymphocyte homing related chemokines in the presence of CT. Further study is required to fully understand the detailed mechanism of regulating the homing factors of LT.

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VIA MEDICA

ORIGINAL PAPER / GYNECOLOGY

Ginekologia Polska
2020, vol. 91, no. 6, 308–312

ISSN 0017–0011

DOI 10.5603/GP.2020.0057

# Circulating omentin-1 levels and inflammation in polycystic ovary syndrome

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## ABSTRACT

**Objectives:** The aim of the study was to analyze interrelation between plasma omentin-1 levels and nutritional status and inflammation in PCOS.

**Material and methods:** A cross-sectional study involving 86 PCOS (47 obese) and 72 Non-PCOS women (41 obese) determined anthropometric parameters and body composition. Serum glucose, insulin and omentin-1, TNF-α, sTNFRs, IL-6 and sR-IL6 were measured in the fasting state.

**Results:** Plasma omentin-1 levels were significantly lower in the PCOS than in the Non-PCOS group and both corresponding normal weight and obese subgroups. In three analyzed least-angle regression (LARS) models the lower plasma omentin-1 levels was associated with PCOS occurrence, higher circulating TNF-α and lower IL-6 levels.

**Conclusions:** Suppressed omentin-1 levels in PCOS are characteristic for this disturbance and proinflammatory cytokines are factors modifying secretion of this adipokine.

**Key words:** omentin-1; inflammation; nutritional status; PCOS

Ginekologia Polska 2020; 91, 6: 308–312

## INTRODUCTION

Omentin-1 is an adipokine, released mainly by stromal-vascular cells of adipose tissue [1–3]. The highest omentin mRNA expression was shown in visceral and pericardial adipose tissues, while in subcutaneous is twenty times lower [3].

It has been shown in an experimental model that omentin-1 stimulates Akt phosphorylation and increases insulin-stimulated glucose uptake [2]. In addition, omentin-1 exerts anti-inflammatory properties, and is also associated with decreased cardiovascular risk [4]. Notably, circulating omentin-1 levels are higher in women than in men [5].

Decreased expression, secretion and circulating levels of omentin-1 were shown in obese subjects [5]. Moreover, negative correlations between omentin-1 concentrations and BMI, waist circumference, circulating leptin and insulin levels and positive with adiponectin levels were observed [5]. In addition, an inhibiting effect of high CRP levels on omentin-1 concentrations was found [6]. It has also been shown that weight loss is followed by omentin-1 level increase, proportional to the improvement in insulin sensitivity accompanied by a decrease of insulin levels [7]. Furthermore, 12-weeks of aerobic training causing decreased fat depots

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without changes in body mass was followed by an increase in circulating omentin-1 levels [8].

The results of studies assessing omentin-1 levels in PCOS are inconsistent. Its lower circulating levels were shown independent from nutritional status and inversely associated with androgens and CRP levels [9]. While, no difference between normal weight PCOS and Non-PCOS women was shown despite higher insulin levels and marked insulin resistance in PCOS group [10]. Contrary, Than et al. [6] observed that metformin therapy increased omentin-1 levels in PCOS women.

Recently, we have suggested an impairment of hormonal stroma adipose tissue function regardless of the nutritional status, secondary to insulin resistance and hyperandrogenism in PCOS [11]. We have also found that low grade inflammation is related to nutritional status, independently from PCOS occurrence [12]. So far, only one study documented negative correlation between levels of omentin-1 and IL-6 and TNF-α in PCOS women in the Asian group [13].

The aim of the study was to analyze interrelation between plasma omentin-1 levels and nutritional status and inflammation in PCOS women.

## MATERIAL AND METHODS

The cross-sectional study involved 86 PCOS women (39 normal weight and 47 obese) with stable body mass during last 3-month period diagnosed in Department of Endocrinological Gynecology from 2013 to 2014. The diagnosis of PCOS was based on Rotterdam ESHRE/ASRM criteria from 2003 [14]. Seventy-two women without PCOS (31 normal weight and 41 obese) constitute the control group. Patients with Cushing's syndrome, thyroid dysfunctions, androgen secreting tumor, and enzyme deficiency (21-hydroxylase in particular), decreased ovary reserves, type 1 and 2 diabetes were not enrolled. Any pharmacological therapy, smoking and alcohol abuse were among the exclusion criteria. The study was conducted after obtaining of the informed consent from each participant. Study protocol was approved by the Ethical Committee of Medical University of Silesia.

Normal weight was defined as body mass index (BMI) from 18.5 to 24.9 kg/m$^2$ and obesity as $\geq$ 30.0 kg/m$^2$. The characteristics of the study groups are presented in Table 1.

All the study women were tested within 3 and 5 days of menstrual cycle. Anthropometric measurements (body mass, height and waist circumference) were performed, and BMI was calculated according to the standard formula. Body composition was assessed by bioimpedance method using Bodystat 1500 (Douglas, Isle of Man). 15 mL samples of venous blood were withdrawn in the morning between 8.00–9.00 a.m., after an overnight fast (16 h). The blood samples were collected according to recommendation of manufacturer of the kits. Serum and plasma samples were stored frozen in –70°C.

### Laboratory procedures

Plasma glucose was estimated by colorimetric methods using the commercially available test kits (Roche, Switzerland). Serum insulin concentration was determined by enzyme-linked immunosorbent assay (ELISA) (DRG Instruments GmbH, Marburg, Germany) with a lower limit of sensitivity of 1.76 μIU/mL and intra- and inter-assay coefficients of variations of 2.2% and 4.4%, respectively. HOMA-IR index was calculated with the standard formula: HOMA-IR = fasting concentration of insulin (μIU/mL) × fasting concentration of glucose (mmol/L)/ 22.5.

ELISA method was also used for measurements of plasma omentin-1 levels (BioVendor, Brno, Czech Republic) with the lower limit of sensitivity of 0.5 ng/mL and intra- and inter-assay coefficients of variations were 3.65% and 4.6%, respectively as well as TNF-α, sTNFR1, sTNFR2, IL-6 and sR-IL-6 (R&D Systems, Michigan, USA) with the lower limit of sensitivity 0.18 pg/mL, 0.77 pg/mL, 0.6 pg/mL, 0.5 pg/mL and 0.88 pg/mL, respectively and intra- and inter-assay coefficients of variations were 14.4% and 18.7% respectively for TNF-α, 3.6% and 2.6% respectively for sTNFR1, 3.7% and 3.5% respectively for sTNFR2, 16.8% and 17.2% respectively for IL-6 and 9.7% and 9.6% respectively for sR-IL-6.

### Statistical analysis

Statistical analyses were performed using STATISTICA 9.0 PL (StatSoft Poland) software and R software environment. There was no missing data in the database. The results are presented as mean values ± standard deviation. Distribution of variables was evaluated by the D'Agostino-Pearson test. Homogeneity of variances was assessed by the Levene test. Quantitative variables were compared with two-way multivariate analysis of variances with Duncan test post-hoc. The assessment of association between variables was done with the multivariate linear regression and the backward stepwise procedure. Outliers were identified based on Cook's distance values. The Cook-Weisberg test was used to test the residuals for heteroskedasticity. Models calculation was performed including evaluation of multicollinearity, which was assessed with the variance inflation factor (VIF). The VIF should not exceed more than five. Goodness of fit of obtained model was assessed with the F test and determination coefficient $R^2$. All results were considered as statistically significant with a p value of $< 0.05$.

## RESULTS

The age of both obese subgroups (PCOS and Non-PCOS) as well as of normal weight subgroups (PCOS and Non-PCOS)

**Table 1.** Study groups and subgroups characteristics'

| | All PCOS (N = 86) | All Non-PCOS (N = 72) | p PCOS vs Non-PCOS | Normal weight PCOS (N = 39) | Normal weight Non-PCOS (N = 31) | p NW PCOS vs NW Non-PCOS | p NW PCOS vs Obese PCOS | p NW PCOS vs Obese Non-PCOS | p NW Non-PCOS vs Obese Non-PCOS | Obese PCOS (N = 47) | Obese Non-PCOS (N = 41) | p Obese PCOS vs Obese Non-PCOS | p Obese PCOS vs. NW Non-PCOS |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Age [year] | 25.4 ± 5.5 | 26.4 ± 5.5 | NS | 23.7 ± 4.5 | 23.8 ± 4.3 | NS | NS | < 0.01 | < 0.001 | 26.8 ± 5.8 | 28.4 ± 5.6 | NS | NS |
| Body mass [kg] | 79.4 ± 26.4 | 78.7 ± 20.4 | NS | 56.9 ± 11.7 | 59.8 ± 7.1 | NS | < 0.001 | < 0.001 | < 0.001 | 97.6 ± 20.4 | 93.1 ± 14.6 | NS | < 0.001 |
| BMI [kg/m$^2$] | 28.6 (20.8–35.7) | 28.5 (22.9–33.5) | NS | 20.6 (19.6–22.7) | 22.4 (21.0–24.0) | NS | < 0.001 | < 0.001 | < 0.001 | 35.1 (31.3–40.2) | 32.9 (30.3–36.7) | NS | < 0.001 |
| Body fat [kg] | 30.2 (15.4–42.6) | 33.3 (19.1–50.4) | NS | 15.0 (12.6–19.7) | 18.1 (14.8–20.6) | NS | < 0.01 | < 0.01 | < 0.01 | 40.6 (33.4–56.3) | 49.4 (37.5–50.2) | NS | < 0.01 |
| Body fat [%] | 38.1 (27.5–45.7) | 40.6 (30.4–48.5) | NS | 26.5 (24.2–31.0) | 30.0 (26.8–33.9) | NS | < 0.001 | < 0.001 | < 0.001 | 44.8 (41.9–51.1) | 46.8 (42.3–51.4) | NS | < 0.001 |
| WC [cm] | 89.8 ± 18.7 | 87.9 ± 18.2 | NS | 72.6 ± 7.3 | 70.5 ± 8.3 | NS | < 0.001 | < 0.001 | < 0.001 | 103.7 ± 12.5 | 101.0 ± 11.3 | NS | < 0.001 |
| Glucose [mmol/L] | 5.1 ± 0.8 | 4.7 ± 0.4 | < 0.001 | 4.9 ± 0.7 | 4.7 ± 0.5 | < 0.01 | NS | NS | NS | 5.3 ± 0.9 | 4.7 ± 0.4 | < 0.01 | < 0.01 |
| Insulin [µIU/mL] | 10.6 (7.8–15.1) | 7.4 (5.9–9.5) | < 0.01 | 8.4 (6.0–10.6) | 6.8 (5.6–8.7) | NS | < 0.01 | NS | NS | 12.9 (9.7–18.6) | 7.8 (6.3–10.0) | < 0.01 | < 0.01 |
| HOMA–IR | 2.3 (1.6–3.2) | 1.5 (1.2–2.0) | < 0.01 | 1.8 (1.2–2.3) | 1.5 (1.1–1.9) | NS | < 0.01 | NS | NS | 2.8 (1.2–4.1) | 1.7 (1.4–2.2) | < 0.01 | < 0.01 |
| Omentin–1 [ng/mL] | 210.5 (149–302.7) | 515.9 (256.3–779.0) | < 0.001 | 178.1 (148.2–220.9) | 484.1 (280.2–729.7) | < 0.001 | < 0.001 | < 0.001 | < 0.001 | 265.9 (179.8–334.0) | 566.4 (243.2–810.5) | NS | NS |
| TNF-α [pg/mL] | 4.4 ± 2.7 | 4.3 ± 2.5 | NS | 2.9 ± 1.3 | 2.6 ± 1.1 | NS | < 0.001 | < 0.001 | < 0.001 | 5.6 ± 2.9 | 5.6 ± 2.5 | NS | < 0.001 |
| sTNFR1 [pg/mL] | 1480 ± 543 | 1368 ± 445 | NS | 1255 ± 507 | 1334 ± 389 | NS | < 0.001 | NS | NS | 1666 ± 505 | 1396 ± 490 | < 0.05 | < 0.001 |
| sTNFR2 [pg/mL] | 3202 ± 932 | 2875 ± 858 | < 0.05 | 3066 ± 769 | 3222 ± 946 | NS | NS | < 0.01 | < 0.01 | 3315 ± 1043 | 2592 ± 667 | < 0.001 | NS |
| IL-6 [pg/mL] | 1.7 ± 1.3 | 1.9 ± 1.0 | NS | 1.0 ± 0.6 | 0.9 ± 0.5 | NS | < 0.05 | < 0.05 | < 0.05 | 2.1 ± 1.2 | 2.2 ± 1.1 | NS | < 0.05 |
| sR-IL6 [pg/mL] | 61.9 ± 27.0 | 53.3 ± 20.9 | NS | 52.6 ± 17.7 | 57.2 ± 24.5 | NS | < 0.05 | NS | NS | 69.7 ± 30.8 | 50.2 ± 17.3 | < 0.01 | < 0.05 |

was similar (Tab. 1). Body mass and BMI did not differ between the corresponding subgroups of PCOS and Non-PCOS.

The serum concentrations of glucose, insulin and HOMA-IR values were significantly higher in the PCOS group than in the Non-PCOS group. As expected, in the obese PCOS subgroup, serum concentration of insulin and HOMA-IR value were significantly higher than in normal weight PCOS subgroup (Tab. 1).

Plasma omentin-1 levels were significantly lower in the PCOS group than in the Non-PCOS group. The lower plasma omenntin-1 levels were shown in normal weight and obese PCOS than in both corresponding Non-PCOS subgroups (Tab. 1).

There were no differences in plasma TNF-α, TNFR1, IL-6 and sR-IL-6 levels between the PCOS and Non-PCOS groups, while TNFR2 levels were significantly higher in the PCOS group. However, plasma TNF-α and IL-6 levels were significantly higher in both obese PCOS and Non-POS than in corresponding normal weight subgroups. The highest sTNFR1 levels were observed in the obese PCOS subgroup. While, sTNFR2 and sR-IL-6 levels were significantly higher in obese than normal weight PCOS subgroups and significantly lower in obese than normal weight Non-PCOS subgroups (Tab. 1).

### Multiple regression analyses

In Table 2, presented are three analyzed Least-angle regression (LARS) models. Numbers denote sequence of switching on of the variable model. The best results, the lowest fault model fit to the data, in each case, was obtained for a set of three variables. The lower plasma omentin-1 levels were associated with PCOS occurrence, higher circulating TNF-α and lower IL-6 levels (Tab. 2).

## DISCUSSION

Numerous studies published in recent years, including ours, suggested that hormonal dysfunction of adipose tissue is an important link in PCOS pathogenesis [11, 15–18]. Obesity, especially visceral, is well known risk factor of PCOS development in genetically predisposed women [19, 20]. Inflammation in visceral adipose tissue is a key factor affecting the release of adipokines [21]. The changes in the profile of circulating adipokines were shown to be associated with the development of insulin resistance and both pituitary and ovary hormonal dysfunction [22].

**Table 2.** The models regression type LARS and the resulting model of the impact of variable on plasma omentin-1 levels

| Variable $y = \log_{10}$(Omentin-1) [ng/mL] | Model I | Model II | Model III | Final model |
|---|---|---|---|---|
| PCOS ± | 1 | 1 | 1 | –0.2432 |
| BMI [kg/m$^2$] | 8 | – | – | – |
| Fat percentage [%] | – | 8 | – | – |
| Waist circumference [cm] | – | – | 8 | – |
| $\log_{10}$(HOMA-IR) | 7 | 7 | 7 | – |
| $\log_{10}$(TNF-α) [pg/mL] | 3 | 3 | 3 | –0.0652 |
| $\log_{10}$(sTNF-R1) [pg/mL] | 5 | 5 | 5 | – |
| sTNF-R2 [pg/mL] | 6 | 6 | 6 | – |
| $\log_{10}$(IL-6) [pg/mL] | 2 | 2 | 2 | 0.0745 |
| $\log_{10}$(sR-IL6) [pg/mL] | 4 | 4 | 4 | – |

The previously published studies have shown lower plasma omentin-1 levels in PCOS women, independently from nutritional status [6, 9, 11]. Contrary to some studies [5, 10] we did not observe the association between plasma omentin-1 levels and anthropometric parameters. This discrepancy is difficult to explain. However, the results of our recently published study suggest that hormonal dysfunction of stromal cells of adipose tissue is the result, but not a cause of insulin resistance development [11]. This hypothesis is supported by the fact that HOMA-IR values in the obese non-PCOS subgroup were below the cut-off point for the insulin resistance. Moreover, Cai et al. [23] revealed that the impaired function of stromal cells of adipose tissue is strongly associated with insulin resistance. This may explain lower omentin-1 levels in the obese, but not in the normal weight PCOS subgroup. Based on our own and other studies [9, 11] we suggest that androgens may be an important factor in explaining disturbances of hormonal function of stromal cells of adipose tissue. Disturbances of omentin-1 synthesis in adipose tissue are not the primary effect of visceral fat accumulation, but rather secondary to the development of insulin resistance and hormonal disturbances [11]. However, inflammation in visceral adipose tissue may be an important cause for decreased omentin-1 synthesis. As it was mentioned above, a single study performed in an Asian PCOS group showed a negative correlation between omentin-1 and IL-6 and TNF-α levels [13]. In accordance with this data, multiple regression analysis in the current study showed a negative impact of TNF-α on omentin-1 levels. However, this association is controversial, as TNF-α levels were significantly lower in the normal weight PCOS group than both obese PCOS and Non-PCOS subgroups. In addition, TNF-α levels were similar in normal weight PCOS and Non-PCOS subgroups. These results are in accordance with our study published over 10 years ago that PCOS is not associated with chronic inflammation per se [24]. Contrary to previously published data, our study regression model suggests that IL-6 may stimulate omentin-1 synthesis. This discrepancy is difficult to explain, but potentially related to the dual, proinflammatory and anti-inflammatory function of IL-6. It should be noted that the proinflammatory properties of cytokines do not correspond to lower circulating omentin-1 levels in normal weigh and obese PCOS showing expected differences in TNF-α and IL-6 levels [12, 25]. Our study suggests that the mechanism of stromal cells dysfunc-

tion in adipose tissue in PCOS is more complex, including impact of insulin resistance, androgens and proinflammatory cytokines. However, these factors may explain lower omentin-1 levels in obese PCOS, it does not fully elucidate the cause of its decreased release in normal weight PCOS. Thus, the relationship between this cytokine and omentin-1 levels requires experimental studies on cell cultures assessing the effect of this cytokine on omentin-1 expression. Although, it cannot be excluded that the decreased concentration of circulating omentin-1 in PCOS women with normal body mass is the result of its' disturbed secretion in ovaries, but not in adipose tissue [26]. Verification of this hypothesis also requires further experimental studies.

The limitation of our study is the size of the study subgroups and the lack of separation of PCOS with normal weight for subgroups with and without metabolic obesity. Moreover, the distribution of body fat and its visceral deposits were not directly assessed using DEXA or CT scanner. Additionally, in our study, the impact of selected adipokines on omentin-1 levels has not been analyzed.

## CONCLUSIONS

Suppressed omentin-1 levels in PCOS are characteristic for this disturbance whereas proinflammatory cytokines are factors modifying secretion of this adipokine.

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VIA MEDICA

ORIGINAL PAPER / GYNECOLOGY

Ginekologia Polska
2020, vol. 91, no. 6, 313–319

ISSN 0017–0011

DOI 10.5603/GP.2020.0060

# The relationship of ovarian endometrioma and its size to the preoperative serum anti-Mullerian hormone level

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## ABSTRACT

**Objectives:** The aim of this study is to evaluate the impact of ovarian endometrioma according to its size on the serum anti-Mullerian hormone (AMH) levels compared to that of other benign ovarian cysts.

**Material and methods:** The current study retrospectively evaluated preoperative serum AMH level and its association to presenting ovarian cyst size which were measured in clinical setting. Women with surgically diagnosed endometrioma or other benign ovarian cysts were included. All patients underwent transvaginal or transrectal ultrasonography to determine the size of the ovarian cysts. Preoperative serum AMH level was checked and evaluated according to histologic type of the cyst, which were endometrioma or other benign ovarian cysts, respectively. Both groups were classified into ≤ 4 cm, > 4 cm and ≤ 8 cm, > 8 cm and ≤ 12 cm, > 12 cm according to the diameter of cyst and analyzed the difference of mean AMH levels in both groups.

**Results:** There was no significant difference in preoperative serum AMH level between the two groups (3.36 ± 2.3 versus 3.76 ± 2.64, p = 0.331). The difference of preoperative AMH levels according to categorized cyst size also was not statistically significant in both groups.

**Conclusions:** Preoperative serum AMH levels were not statistically different between endometrioma and other benign ovarian cyst groups and were not related to the size of endometrioma.

**Key words:** AMH; endometriosis; endometrioma; ovarian cyst

Ginekologia Polska 2020; 91, 6: 313–319

## INTRODUCTION

Anti-Müllerian hormone (AMH) is a dimeric glycoprotein that belongs to the transforming growth factor-beta family [1]. It is involved in the regression of the Müllerian ducts during male fetal development. In the female, AMH is solely produced by the granulosa cells of primary, preantral and small antral follicles in the ovaries [2].

Clinical aspects of AMH have some exclusive characteristics; the serum AMH level is closely related with age, with insignificant intra- and inter-cycle variation throughout menstrual cycles [3, 4]. Because of such characteristics, AMH has been significantly used in several clinical practices such as assisted reproductive technologies [5, 6], prediction of menopause and diagnosis of PCOS [7–10]. Yet, while serum AMH level may be the best marker of ovarian reserve, the utility as a predictive marker for live births or timing of menopause is not reached to the definite conclusions [7].

In gynecological perspective with interests in clinical use of AMH, the impact of a benign ovarian cyst on physiological serum AMH level is also one of the clinical issues with indefinite conclusion [11–13]. Such debate could be considered in two points; influence of surgery for an ovarian cyst and that of an ovarian cyst itself on the serum AMH level.

Regarding the influence of surgery on ovarian cysts and serum AMH level, it is widely accepted that ovarian reserve measured with serum AMH level would be reduced after ovarian cystectomy [14–16]. Henes et al. reported that serum AMH level was significantly decreased after surgery on a follicular cyst and endometriosis, but not on dermoid and other cysts [15].

When considering the influence of endometrioma on the serum AMH level, several subordinate concepts are needed to be pondered: the stage, laterality and size of endometrioma. Still, conflicting results are being observed; some reports have suggested that the serum AMH level is relatively low in

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the endometrioma group [17, 18], but the opposite findings have been also reported [19, 20]. Karadag et al. [17] reported lower AMH levels in patients with bilateral endometrioma than patients with unilateral endometrioma and lower AMH levels in women with deep infiltrating endometriosis. Similarly, Uncu et al. [18] found that endometrioma patients have lower AMH levels and antral follicle count when compared to the control and suggested that the presence of endometrioma itself is associated with a reduction in ovarian reserve. On the other hand, Kim et al. [19] suggested that the serum AMH level were not different between the endometrioma and teratoma groups, but in age-body mass index matched stage IV endometriosis, the serum AMH level was lower in the endometrioma group. Despite the ongoing debate, general consensus is that higher stage and bilaterality of endometrioma are more likely to result low AMH [21–23].

Expanding the issue to the relationship of size of endometrioma and serum AMH level, the controversies have been either insignificant or negative relationship of the two. However, the recent study of Marcellin et al. [20] has found strikingly positive association between the size of endometrioma and serum AMH level, introducing novel understanding of physiological characteristics of endometrioma and interpreting the associated AMH level. Currently, valuable scholarly investigations on AMH concentrations according to the size of endometrioma have been more complicated to be answered.

The aim of this study is to evaluate the impact of ovarian endometrioma according to its size on the serum anti-Mullerian hormone (AMH) levels compared to that of other benign ovarian cysts.

## MATERIAL AND METHODS

### Patients

This retrospective study was conducted at Department of Obstetrics and Gynecology, Pusan National University Hospital. Women with surgically diagnosed endometrioma between October 01, 2012 and October 01, 2019 were included as the study group. The exclusion criteria were prior ovarian surgery, irregular menstrual periods, the presence of polycystic ovary syndrome, hyperprolactinemia or abnormal thyroid function test (TFT), and medication history with dysmenorrhea management such as GnRH analogues, oral contraceptives or progestins during the past 3 months to the recruitment. The control group was comprised of women with benign ovarian cysts other than endometrioma. The same exclusion criteria were applied to the controls. BMI was calculated as the patient's weight in kilograms divided by her height in meters squared. All patients underwent laparoscopic or laparotomic ovarian cystectomy. All specimens obtained intraoperatively were submitted for pathologic examination, and pathologic confirmation was done in all cases. All patients agreed and provided informed consent forms indicating that their medical records were to be used for the study. The study was approved by the ethics committee of Pusan National University Hospital.

### Measurement of ovarian cyst size

All patients underwent transvaginal or transrectal ultrasonography to determine the size of the endometriomas or other benign ovarian cysts using a 5–9 MHz transvaginal transducer or transrectally for virgin patients on the day before surgery (Voluson E6 General Electric, Milwaukee, Wauwatosa, WI, USA). The cyst size was recorded as the average of the largest and shortest diameters in centimeters. Each cyst diameters were measured vertically from outer-membrane to the opposite outer-membrane. Bilateral cysts were recorded as the sum of the two cyst sizes.

### Assay of AMH and inflammatory markers

All of women underwent blood sampling for preoperative AMH measurements and inflammatory markers within a month prior to surgery. Blood samples were obtained from patients after 8hrs of overnight fast, including following measurements, using Roche Modular DP (Tokyo, Japan): complete blood cell count including number of white blood cells (WBC), percentages of segmented_neutrophils and lymphocytes and segmented neutrophil/lymphocyte (N/L) ratio. Serum AMH assay was performed using an anti-Mullerian hormone/Mullerian inhibiting substance Enzyme Immuno Assay (AMH/MIS EIA) kit (Immunotech version, Beckman Coulter, Marseille, France). The coefficients of variation of intra-assay and inter-assay were 12.3% and 14.2%, respectively. The distribution of AMH values for two or three days of the physiological cycle according to the patient's age is determined by using 5%, 10%, 25%, 50%, 75%, 90%, 95%, and mean values according to age was calculated [24].

### Statistics

All statistical data was organized into a computerized database. Variables were evaluated for clinical significance using chi-square test and Fisher's exact test for categorical variables or the independent t-test for continuous variables, where appropriate. One-way ANOVA was used to compare preoperative serum AMH levels according to categorized cyst sizes in the endometrioma group and control group. Pearson correlation coefficient was used for correlation between preoperative AMH level, age, ovarian cyst size and inflammatory markers. Using multiple linear regression analysis, factors affecting preoperative serum AMH level were studied. The statistical analysis was performed SPSS version 22.

## RESULTS

The mean age of the patients was higher in the endometrioma group compared to the control group, which

were 30.45 ± 5.99 years and 28.09 ± 7.55 years, respectively (p = 0.034). Parity was lower in the endometrioma group than in the control group (0.09 ± 0.35 versus 0.30 ± 0.71, p = 0.033) and showed a regular menstrual cycle compared to the control group (79.66% vs 20.34%, p < 0.001). In addition, dysmenorrhea in the endometrioma group were higher than the control group (82.2% vs 59.38%, p = 0.001). There were no significant differences in preoperative serum AMH level between the two groups (3.36 ± 2.3 vs 3.76 ± 2.64, p = 0.331). Serum WBC count and N/L ratio were significantly higher in endometriosis group than in control group (Tab. 1).

Table 2 shows the difference of preoperative AMH levels according to cyst size in endometrioma and control group. The groups were classified into ≤ 4 cm, > 4 cm and ≤ 8 cm, > 8 cm and ≤ 12 cm, > 12 cm according to the cyst size. The difference of preoperative AMH levels accord-

**Table 1.** Basic characteristics of the patients in control and endometrioma group

| | Overall (n = 182) | Control (n = 64) | Endometrioma (n = 118) | p value |
|---|---|---|---|---|
| Age | 29.62 (6.66) | 28.09 (7.55) | 30.45 (5.99) | 0.034 |
| BMI | 21.90 (4.30) | 22.51 (4.58) | 21.56 (4.12) | 0.154 |
| Smoking history* | | | | 1.000 |
| Never | 175 (96.15) | 62 (96.88) | 113 (95.76) | |
| Current | 7 (3.85) | 2 (3.12) | 5 (4.24) | |
| Previous | 0 (0.00) | 0 (0.00) | 0 (0.00) | |
| Bilaterality* | | | | 0.310 |
| No | 128 (70.33) | 48 (75) | 80 (67.8) | |
| Yes | 54 (29.67) | 16 (25) | 38 (32.2) | |
| Past history* | | | | 0.015 |
| None | 168 (92.31) | 59 (92.19) | 109 (92.37) | |
| HTN | 3 (1.65) | 2 (3.12) | 1 (0.85) | |
| DM | 1 (0.55) | 0 (0.00) | 1 (0.85) | |
| Hepatitis | 2 (1.1) | 2 (3.12) | 0 (0.00) | |
| Thyroid disease | 7 (3.85) | 0 (0.00) | 7 (5.93) | |
| Cancer | 1 (0.55) | 1 (1.56) | 0 (0.00) | |
| Gravidity | 0.37 (0.86) | 0.52 (1.04) | 0.30 (0.74) | 0.139 |
| Parity | 0.16 (0.51) | 0.30 (0.71) | 0.09 (0.35) | 0.033 |
| OCs* | | | | 1.000 |
| Never | 175 (96.15) | 62 (96.88) | 113 (95.76) | |
| Current | 4 (2.2) | 1 (1.56) | 3 (2.54) | |
| Previous | 3 (1.65) | 1 (1.56) | 2 (1.69) | |
| Menstruation* | | | | < 0.001 |
| Regular | 129 (70.88) | 35 (54.69) | 94 (79.66) | |
| Irregular | 53 (29.12) | 29 (45.31) | 24 (20.34) | |
| Dysmenorrhea* | | | | 0.001 |
| No | 47 (25.82) | 26 (40.62) | 21 (17.8) | |
| Yes | 135 (74.18) | 38 (59.38) | 97 (82.2) | |
| Menarche | 13.32 (1.48) | 13.19 (1.61) | 13.40 (1.41) | 0.381 |
| AMH (ng/mL) | 3.50 (2.63) | 3.76 (2.64) | 3.36 (2.63) | 0.331 |
| WBC (× 103/uL) | 6.66 (2.03) | 6.23 (1.56) | 6.90 (2.22) | 0.019 |
| Segmented_neutrophil (%) | 59.35 (10.65) | 58.07 (9.93) | 60.05 (11.01) | 0.218 |
| Lymphocyte (%) | 31.67 (9.41) | 32.83 (8.78) | 31.03 (9.71) | 0.206 |
| N/L | 2.46 (3.46) | 1.99 (0.99) | 2.71 (4.22) | 0.080 |
| Cyst size (cm) | 6.90 (4.03) | 7.59 (4.10) | 6.52 (3.96) | 0.091 |

Data are presented as the means (SD); * values are presented as the number of patient (%); p value by independent t-test for continuous variable; * Chi-square test or Fisher's exact test for categorical variable; BMI — body mass index; HTM — hypertension; DM — diabetes mellitus; OCs — oral contraceptives; AMH — anti-Mullerian hormone; WBC — white blood cell; N/L — segmented neutrophil/lymphocyte ratio

**Table 2.** Comparisons of serum AMH level according to cyst size in c`ontrol and endometrioma group

| Cyst size (cm) | ≤ 4 | > 4 & ≤ 8 | > 8 & ≤ 12 | > 12 | p value |
|---|---|---|---|---|---|
| Overall (n = 182) | 41, 3.39 (2.68) | 89, 3.62 (2.68) | 35, 3.50 (2.47) | 15, 3.27 (2.89) | 0.948 |
| Control (n = 64) | 9, 5.04 (3.75) | 34, 3.82 (2.78) | 15, 3.11 (1.62) | 6, 3.08 (1.50) | 0.336 |
| Endometrioma (n =1 16) | 32, 2.92 (2.16) | 55, 3.49 (2.64) | 20, 3.79 (2.97) | 9, 3.39 (3.63) | 0.675 |
| Data are presented as the number of patients, means (SD) | | | | | |

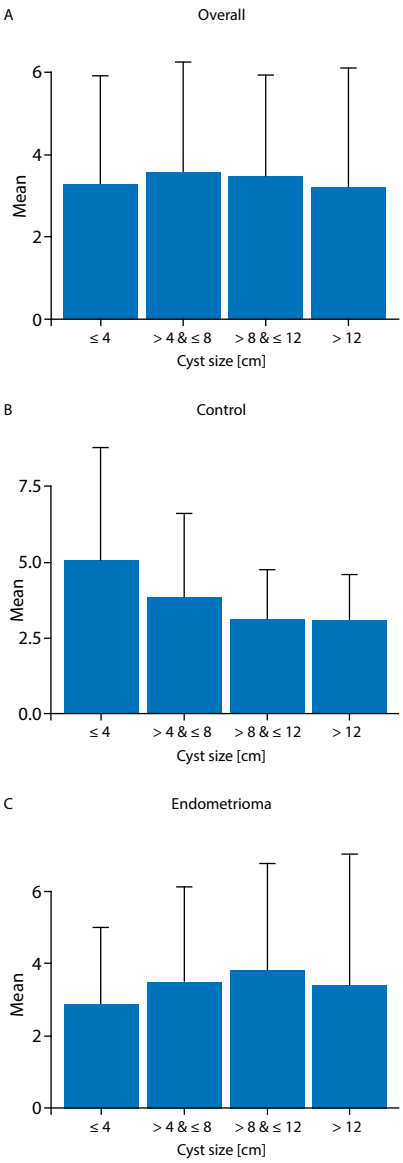


**Figure 1.** The value of the serum AMH according to average ovarian cyst size; A. Overall group; B. Control group including benign ovarian cyst except endometrioma; C. Endometrioma group

ing to categorized cyst size was not statistically significant in both groups (p = 0.336, p = 0.675, respectively), regardless of bilaterality (Fig. 1).

Table 3 shows the results of the correlation between inflammatory markers and preoperative AMH levels. Pearson correlation coefficient showed only age factors have a significantly negative correlation (Rho value = –0.357 with p < 0.0001). Serum WBC count and N/L ratio had Rho values of –0.009 (p = 0.907) and –0.022 (p = 0.766), respectively.

**Table 3.** Correlations between preoperative AMH and other parameters including all groups

| (n = 182) | Rho value | p value |
|---|---|---|
| Age [years] | –0.357 | < 0.0001 |
| Average of cyst size [cm] | –0.051 | 0.492 |
| N/L ratio | –0.022 | 0.766 |
| Number of WBC | –0.009 | 0.907 |

AMH — anti-Mullerian hormone; N/L — segmented neutrophil/lymphocyte; WBC — white blood cell

The result of multiple linear regression analysis including age, cyst size, serum WBC count and N/L ratio showed non-significant statistical correlation except age (coefficient β = –0.15, p < 0.0001).

## DISCUSSION

The surgery-related decrease of ovarian reserve in endometriosis is relatively well-established finding [2, 15, 16, 18]. However, the impact of ovarian endometrioma itself on ovarian reserve — one of the most important preoperative characteristics to decide further treatment method — showed inconsistent results. Uncu et al. [18] reported that women with endometrioma had lower AMH levels and antral follicle count than controls, and Pacchiarotti et al. [25] also observed the negative effect of endometriosis on the ovarian reserve, especially in cases of severe endometriosis. The possible underlying mechanism suggested by the authors was that increased peritoneal macrophages in endometriosis could have caused substantial damage to ovarian tissue and oxidative stress, inducing oocyte degeneration and apoptosis by disturbing the meiotic spindle [25, 26]. Yet, in these studies, the control groups comprised women of reproductive age who did not have any ovarian cysts [18]. Thus, it would be unclear to conclude whether such reduced serum AMH level in the endometrioma group was caused specifically by endometrioma or generally by the presence of cystic lesion on the ovary. When the control group had benign ovarian cysts, some studies have reported the insig-

nificant difference in AMH level between the control and the patient group with endometrioma [12, 19]. More detailed evaluation and analysis according to the characteristics of endometrioma itself and suitable control group are required to interpret AMH level in endometrioma patients.

Recently, Marcellin et al. [20] evaluated serum AMH levels in endometrioma patients according to the size of the cyst; interestingly, they reported that serum AMH levels in women with no prior history of surgery for endometrioma increased with the cyst size of endometrioma. In the control group, non-endometrioma benign ovarian cyst group, serum AMH levels was not related with the size of cyst. The authors concluded that serum AMH level had positive correlation with endometrioma size and was not affected by laterality of endometrioma or deep infiltrating endometriosis, which was strikingly different from previous literature.

In this study, to investigate such inconclusive effect of endometrioma itself on serum AMH level according to its size, we compared the preoperative AMH level between endometrioma and other benign ovarian cyst group, conducting subgroup analysis according to corresponding ovarian cyst size.

First, we observed that the effect of benign ovarian cyst on serum AMH level was not different between the endometrioma and the other benign ovarian cyst group. Such result of ours was in agreement with the study of Streuli I et al. [13], in which they reported that the serum AMH levels was not different between women with endometriosis and women with benign gynecological condition (endometrioma group, $3.6 \pm 3.1$ ng/mL and control group, $4.1 \pm 3.4$ ng/mL, $p = 0.06$). Therefore, preoperative serum AMH level seems to be similar between endometrioma and other benign ovarian cysts, as previously known [13, 20].

Next, we compared the relationship of ovarian cyst size and serum AMH level. In our study, there was no statistical difference between endometrioma size and serum AMH level, which was inconsistent with the results of Marcellin et al. However, except for the endometrioma with size of more than 12 cm, the serum AMH level tended to be increased with the increasing cyst size. Additionally, we have run the same statistical analysis on unilateral endometrioma and bilateral endometrioma separately, and, interesting enough, the similar pattern was observed (data not shown). Despite statistical insignificance, in unilateral endometrioma, serum AMH level tended to be increased with increasing cyst size up to 6 cm and dropped thereafter; in bilateral endometrioma, the same tendency was observed up to the cyst size of 12 cm. At this point, we supposed that serum AMH level increased with increasing endometrioma size up to a certain limit — in this study, the upper limit was 12 cm in diameter when the unilateral and bilateral endometrioma were combined — and became insignificant thereafter. Though further statistical analysis on subdivided sizes of endometrioma is warranted, based on the current results, the size of endometrioma might have affected the serum AMH level, not necessarily reflecting the ovarian reserve, which concurs with the results of Marcellin et al. [20].

Marcellin et al. suggested three hypotheses to explain their results [20]. First is selection bias. In case of women with low AMH levels, surgical management tended to be avoided. Second, they suggested that there may be increased secretion of AMH into the circulation by the ovaries as the size of the endometrioma increases; inflammation and neoangiogenesis in endometriosis could boost the local blood clearance from the ovaries. Third, the toxicity of endometrioma on the ovarian reserve may contribute improved primordial follicular stimulation and consequently an increase in serum AMH levels.

Of the three hypotheses of Marcellin et al. [20], regarding the selection bias, we also excluded infertile patients, suggesting the potential of selection bias may exist. Streuli et al. [13] excluded infertile patients who were more likely to have lower basal AMH in their study as well, and they reported similar results to ours. Considering the effect of infertile patient, Kim et al. [19] reported that the preoperative serum AMH was not different between endometrioma and mature cystic teratoma, including infertile patients in their subject group (endometrioma group 9.8% and control group 7.8%, p = not significant, respectively); additionally, in case of stage IV endometriosis, the serum AMH level was lower in the endometrioma group. Thus, regardless of including infertile patient to the subject group, the basal serum AMH level was not significantly different between patients with endometrioma or other benign ovarian cysts. Still, more detailed and larger studies are needed considering infertility history and endometrioma severity.

For evaluating the effect of inflammation, we analyzed the relationship of inflammation markers; WBC count and N/L ratio. The results showed no statistical relevance. However, it is hard to define that the number of serum WBCs and N/L ratio reflected the inflammatory environment of peritoneal cavity related with endometrioma. It is necessary to analyze the inflammatory marker of peritoneal fluid.

Last, as reported elsewhere, an increase in endometrioma size might cause increasing toxicity on the ovarian reserve, and short-term exposure of human ovarian follicles to cyclophosphamide metabolites seems to promote follicular activation in vitro [27, 28]. However, such studies were conducted in vitro, and the toxicity of cyclophosphamide metabolite and endometrioma might differ in clinical setting.

The current study has several limitations. First, the number of patients included in this study was relatively small. However, data were collected in a single institute, and all patients were firmly diagnosed by laparoscopic surgery.

We clearly excluded minimal or mild endometriosis patients in control group by laparoscopy, leading the consistency of data to be quite valuable. Second, the mean age of control group was significantly lower than the endometrioma group. Despite the slightly higher mean age, the mean AMH of the endometrioma group was comparable to the control group. Thus, we could logically conclude that the preoperative AMH were not different between the endometrioma and control groups. Lastly, since the current study is retrospective, all statistical analysis was inevitably performed using previously measured data; yet, Marcellin et al. [20] adopted the sum of the largest diameters for bilateral ovarian cysts in their study of increasing serum AMH level with endometrioma size, when, in previous literature, women with bilateral ovarian cysts had been reported to have lower serum AMH levels, irrespective of the nature of the cyst [12]. In the current study, regardless of bilaterality of ovarian cysts, increasing tendency of serum AMH level with ovarian cyst size was observed, giving the important perspective of relationship between serum AMH level and the size of cyst in endometrioma.

## CONCLUSIONS

The preoperative serum AMH levels were not statistically different between the endometrioma and other benign ovarian cyst groups and were not related to the size of endometrioma. Nevertheless, except for the cysts larger than 12 cm in diameter, the serum AMH level tended to have positive relationship with increasing endometrioma size. With further analyses with larger number of patients and more suitable inflammatory markers, future findings on the association between serum AMH level and endometrioma size could innovatively suggest proper interpretation of preoperative serum AMH level and clear management direction for treatment in endometrioma patients.

### *Conflict of interest*

None of the authors have direct or indirect conflict interest associated with publishing the article.

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VIA MEDICA

ORIGINAL PAPER / GYNECOLOGY

Ginekologia Polska
2020, vol. 91, no. 6, 320–323

ISSN 0017–0011

DOI 10.5603/GP.a2020.0063

# Recurrent pregnancy loss and metabolic syndrome

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## ABSTRACT

**Objectives:** The aim of this study was to evaluate the frequency of metabolic syndrome (MetS) and its components in patients with unexplained recurrent pregnancy loss (RPL).

**Material and methods:** A cross-sectional study was held including 115 patients with unexplained RPL who were referred to a tertiary center between December 2018 and December 2019. In the study, MetS was classified according to The National Cholesterol Education Program (NCEP) Adult Treatment Panel III (ATP III) criteria on the basis of metabolic risk factors. Frequency of MetS in the patients with unexplained RPL was investigated. The relationship between miscarriage rate and metabolic risk factors was also evaluated.

**Results:** According to our study the percentage of MetS in patients with unexplained RPL was 24.4%. When evaluated according to different age groups, it was 18.4% in patients aged 20–29 years, and it was 27.8% in patients aged 30–39 years. At least having one of its components were high (82.6%) in all patients with unexplained RPL.

**Conclusions:** The percentage of MetS or of at least having one of its components were high in patients with unexplained RPL. Increased number of having MetS components were associated with increased miscarriage rate.

**Key words:** recurrent pregnancy loss; miscarriage; metabolic syndrome; HDL; anticoagulant

Ginekologia Polska 2020; 91, 6: 320–323

## INTRODUCTION

Pregnancy loss is the most common complication of pregnancy, and RPL is defined as two or more failed clinical pregnancies as documented with histopathologic examination or ultrasonography [1]. This problem affects 1% to 5% of all couples trying to conceive [2]. Causes of RPL have been attributed to either endocrine, genetic, structural, immune, or infective factors. In addition to these factors, the causes of almost half of the cases remain unexplained [3]. MetS is a pandemic health problem that includes some clinical findings such as hyperglycemia, dyslipidemia, abdominal obesity, and hypertension which are metabolic risk factors for both cardiovascular disease and type 2 diabetes [4]. It has been shown that rare complications of pregnancy such as preeclampsia, and small for gestational age infancy are variously associated with the MetS [5]. Hyperglycemia and hypertension, which are components of the MetS, can affect the vascular structure. Since the placenta is a vascular structure, any condition that may affect the vascular system may adversely affect the pregnancy outcomes. We suggest that MetS may be an etiological factor of unexplained RPL, hence we want to evaluate the frequency of MetS and its components in patients with RPL.

## MATERIAL AND METHODS

This prospective cross-sectional study was conducted with a total of 115 patients with unexplained RPL who were referred to Harran University Hospital in Sanliurfa, Turkey, which is a tertiary referral center, between December 2018 and December 2019. The study was approved by the Local Institutional Research Ethics Board. The characteristics of patients were shown in Table 1. Inclusion criteria were patients who lost two or more consecutive pregnancies before the 20$^{th}$ week of gestation with or without a previous live birth. All participants signed written informed consent prior to participating in the study. The clinical and obstetric history of participants was obtained. To investigate the etiology of RPL, all women were examined with a transvaginal ultrasound to identify congenital uterine abnormalities and intrauterine pathologies, and where necessary for confir-

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**Table 1. Characteristics of patients recruited for the study**

| | Mean | Min–Max |
|---|---|---|
| Age [years] | 30.30 ± 6.64 | 18–45 |
| Gravidy | 5.35 ± 2.41 | 2–13 |
| Miscarriage rate | 3.28 ± 1.39 | 2–8 |
| Systolic blood pressure [mm Hg] | 117.89 ± 10.02 | 100–170 |
| Diastolic blood pressure [mm Hg] | 74.76 ± 7.22 | 55–90 |
| Body mass index [kg/m$^2$] | 26.28 ± 4.25 | 19–42 |
| Waist circumference [cm] | 83.14 ± 13.63 | 56–120 |
| HDL- cholesterol [mg/dL] | 50.03 ± 12.89 | 30–88 |
| Triglyceride [mg/dL] | 129.97 ± 75.99 | 41–452 |
| Fasting glucose [mg/dL] | 93.86 ± 10.93 | 75–125 |

Data were presented as mean ± standart deviation

mation, patients underwent a sonohysterography. Blood samples of participants were taken to determine possible endocrinological reasons for RPL, such as thyroid disorders, diabetes mellitus (DM), and hyperprolactinemia. Anticardiolipin Ig G and M, and levels of lupus anticoagulant were also evaluated to detect patients with Antiphospholipid Syndrome (APS). APS was diagnosed in accordance with the recommendations of international consensus criteria [6]. Maternal and paternal chromosome assessments were performed. In our clinic, these tests are routinely performed to clarify the etiology of RPL. The study was planned prospectively in order to avoid any problems regarding records of anthropometric measurements and tests. The study exclusion criteria included: Current pregnancy, smokers, alcohol consumption, maternal or paternal chromosome abnormalities, anatomical abnormalities, patients with APS, systemic diseases such as DM, hyperprolactinemia, thyroid dysfunction, chronic hypertension, systemic lupus erythematosus. Height, weight, waist circumference and blood pressure (BP) were measured by trained medical personnel. Body mass index (BMI) was calculated by weight (kg) divided by the height (m$^2$). After 5 minutes of rest, arterial BP was measured on the right upper arm by a semiautomatic oscillometric device. Fasting glucose, high-density lipoprotein, and triglycerides were evaluated from blood as part of a overall health assessment. MetS was diagnosed based on The NCEP ATP III as the presence of any three of the following five traits: 1) Abdominal obesity, defined as a waist circumference ≥ 102 cm (40 in) in men and ≥ 88 cm (35 in) in women. 2) Serum triglycerides ≥ 150 mg/dL (1.7 mmol/L) or drug treatment for elevated triglycerides. 3) Serum HDL-C < 40 mg/dL (1 mmol/L) in men and < 50 mg/dL (1.3 mmol/L) in women or drug treatment for low HDL-C. 4) BP ≥ 130/85 mmHg or drug treatment for elevated BP. 5) Fasting glucose (FG) ≥ 100 mg/dL (5.6 mmol/L) or drug treatment for elevated blood glucose [7].

### Statistical analysis

The Statistical Package for Social Sciences (SPSS 22.0; SPSS Inc., Chicago, IL) was used in all statistical analyses. Application of the Levene's test revealed that data distributions were normal. Results were expressed as means ± standard deviations. Pearson correlation was done. The mean values of the the groups were analyzed using Independent Sample T Test. The values were calculated at level of ($p < 0.05$) significance.

## RESULTS

Among the study group, the majority were in the age group of 30–39 years (54 patients, 47%), followed by 20––29 years (49 patients, 42.6%), 40–49 years (9 patients, 7.8%) and the patients aged below than 20 years were minimum in number (3 patients, 2.6%). The percentage of the MetS diagnosed using the ATP III criteria was 24.4% (28 of 115). The age adjusted percentage of MetS was calculated. It was 18.4% in the age group of 20–29 years, and it was 27.8% in the age group of 30–39 years. There were 3 patients aged below 20 years, MetS was not seen in this group. There were 9 patients in the age group of 40–49 years. MetS was seen in four of them. Because of the low number, percentages were not calculated. There was a negative correlation between HDL-C levels and miscarriage rate ($r = –0.295$, $p < 0.01$). Hyperglycemia and age were also associated with miscarriage rate ($r = 0.277$, $p < 0.01$; $r = 0.272$, $p < 0.01$ respectively). Additionally, an association had been seen between the presence of increased number of metabolic risk factors and miscarriage rate ($r = 0.239$, $p < 0.05$). We found that 27.8%, 30.4%, and 24.4% of the patients had at least 1, 2, or ≥ 3 metabolic risk factors, respectively. At least one metabolic risk factor was seen in 82.6% of patients. The frequency of individual components of the MetS in patients with RPL are presented in Table 2. Low HDL-C and abdominal obesity were the most frequent components of the MetS in patients with RPL. Furthermore, patients were divided into two groups according to whether they had each metabolic risk factor or not, and evaluated in terms of miscarriage rate (Tab. 3). Low HDL-C group was associated with the increased miscarriage rate. It was statistically significant.

## DISCUSSION

In this study we evaluated the percentage of MetS under the ATP III definition in the group of patients with unexplained RPL. We observed that the percentage of MetS or of at least having one of its components were high in these patients. Kozan et al. [8] studied the prevalence of the MetS in the adult Turkish population and they found that the prevalence was 9.6% in women aged 20–29 years, and 29.7 % in women aged 30–39 years. Soysal et al. [9] also studied the prevalence of MetS and its components among

**Table 2.** Frequency of individual components of the MetS in patients with RPL

| All RPL Patients | | |
|---|---|---|
| | **Frequency** | **Percentage** |
| Hyperglycemia | 28/115 | 24.35 |
| Hypertriglyceridemia | 30/115 | 26.09 |
| Abdominal obesity (Waist circumference ≥ 88 cm) | 51/115 | 44.35 |
| Low HDL cholesterol | 67/115 | 58.26 |
| High blood pressure | 15/115 | 13.04 |
| **RPL Patients with MetS** | | |
| | **Frequency** | **Percentage** |
| Hyperglycemia | 17/28 | 60.71 |
| Hypertriglyceridemia | 19/28 | 67.86 |
| Abdominal obesity | 23/28 | 82.14 |
| Low HDL cholesterol | 26/28 | 92.86 |
| High blood pressure | 5/28 | 17.86 |

**Table 3.** The effects of presence of each metabolic risk factors on miscarriage rate

| | | **Misscariage Rate** | **p*** |
|---|---|---|---|
| HDL Cholesterol | < 50 mg/dL<br>≥ 50 mg/dL | 3.59 ± 1.51<br>2.83 ± 1.06 | **< 0.01** |
| Triglyceride | ≥ 150 mg/dL<br>< 150 mg/dL | 3.17 ± 1.45<br>3.32 ± 1.47 | > 0.05 |
| Fasting Glucose | ≥ 100 mg/dL<br>< 100 mg/dL | 3.64 ± 1.68<br>3.16 ± 1.27 | > 0.05 |
| Abdominal Obesity | ≥ 88 cm<br>< 88 cm | 3.55 ± 1.55<br>3.06 ± 1.21 | > 0.05 |
| High Blood Pressure | ≥ 130/85 mmHg<br>< 130/85 mmHg | 2.80 ± 0.94<br>3.35 ± 1.43 | > 0.05 |

Data were presented as mean ± standart deviation
*Independent Samples T Test

the young adults. They observed that the prevalence of MetS was 7.5% among 20 and 29 years old women and 24% among 30 and 39 years old women. It is known that MetS is more common in 30–39 years old people than in 20–29 years old people. Therefore, it is important to take notice that the percentage of MetS was 18.4% in 20–29 years old group in our study. Withal, according to our knowledge this is the first study to evaluate the percentage of MetS in patients with unexplained RPL.

Our study also examined the relationship between each component of MetS and the miscarriage rate. The increased miscarriage rate was statistically significant in patients with low HDL-C. Kozan et al. [8] reported the most prevalent component of the MetS was abdominal obesity in women of Turkish adults, but we observed that low HDL-C was the most common component in our study. The percentage of low HDL-C was 92.86% (26/28) in the patients with MetS with RPL, and it was 58.26% (67/115) in the whole group. As outlined previously, within the whole group there were 3 patients aged below 20 years. Although their ages were below 20 years, two of them had two metabolic risk factors. The first patient had abdominal obesity and low HDL-C. The other one had hyperglycemia and low HDL-C. The common metabolic risk factor was low HDL-C. It is important to remember that low HDL-C is associated with vascular endothelial dysfunction [10]. Although the number of patients is statistically insignificant, we suggest that low HDL-C may predict the early onset of events affecting the vascular structure, therefore it may be an early sign of poor pregnancy outcomes including unexplained RPL.

In the literature, there are studies about the MetS and adverse pregnancy outcomes, Hooijschuur et al. [5] suggested that different clinical manifestations of placental syndromes such as preeclampsia and small for gestational age infancy are variously associated with MetS. Murphy et al. [11] reported that risk ratios and prevalence rates for MetS increased in preeclamptic women at 1 year and 3 years after birth. All components of MetS are associated with oxidative stress, inflammation, and endothelial dysfunction [12], withal 82.6% of patients in our study have one or more metabolic risk factors. Fortunately, endothelial dysfunction is reversible, and treating metabolic risk factors such as hypercholesterolemia and hypertension results in improved endothelial function resulting in restoration of vascular function. New guidelines lower the definition of high blood pressure, and it is accepted as ≥ 130/80 mmHg, in order to prevent negative outcomes of hypertension [13]. Therefore, regulating the tension below 130/80 mmHg may be appropriate for patients with unexplained RPL.

Pregnancy is a procoagulant state, and MetS is associated with idiopathic venous thromboembolism [14], therefore patients with MetS are at high risk of developing thromboembolic disorders during pregnancy [15]. In cases of MetS, anticoagulant therapy from the time of conception may have beneficial effects in these patients. Published data specific to the use of anticoagulant agents in pregnant patients with MetS are needed. MetS is also associated with higher proinflammatory mediators, and heparin has a variety of antiinflammatory potentials. Therefore, some pregnancy complications including unexplained RPL may benefit from anticoagulant therapy such as heparinoids [16]. Although the anticoagulant therapy is not recommended in patients with unexplained RPL [17, 18], we suggest that using anticoagulant therapy may be useful because of high percentage of MetS in these patients, both for treatment and prevention of thromboembolic disorders. Evaluation of these patients only through the window of obstetrics may be insufficient, so a multidisciplinary team approach may be

suitable. Healthier lifestyle changes, treating hypercholesterolemia, treating hypertension according to ACC/AHA [13], and anticoagulant therapy may be appropriate for patients with unexplained RPL. This study has some limitations. It has a cross sectional design, and the sample size of the study is low and data were obtained from a single center. Multi-center studies with more patients may be useful to advance this area of research.

In conclusion, this study may be significant for many reasons. First, MetS and related conditions may play a role in the etiology of unexplained RPL, therefore treating metabolic risk factors may benefit pregnancy outcomes. Secondly, it may be possible to raise awareness of these patients in terms of early cardiovascular risk factors that may occur in the future, thus preventing future complications.

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ORIGINAL PAPER / GYNECOLOGY

Ginekologia Polska
2020, vol. 91, no. 6, 324–330

ISSN 0017–0011

DOI: 10.5603/GP.a2020.0002

# The impact of concurrent HPV infections on the presentation of high grade cervical intraepithelial lesions

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## ABSTRACT

**Objectives:** We investigate how concurrent high-risk (hr) HPV (human papillomavirus) genotypes affect CIN2-3 risk and evaluate the relationship of different genotype combinations with cervical epithelial lesions.

**Material and methods:** This study included HPV positive patients between the ages of 30 and 60 who underwent liquid-based cervical smears and HPV screening through community-based, cervical cancer screening programs between June 2015 and June 2017. The impact of the increase in hrHPV types was calculated by estimating how it changed the odds ratio of CIN2-3 risk.

**Results:** The rate of multiple concurrent HPV infections was 48.7% in the CIN2-3 group and 58.4% in the CIN1 group. Among patients in the CIN2-3 and CIN1 groups, the most common HPV coinfection was respectively HPV 16+31 and HPV 16+51. The HPV 51 ratio in CIN1 patients was 28.9% and the HPV 51 ratio in the CIN2-3 patient was 6.6%. With every increase in the number of hrHPV infection types, the frequency of CIN2-3 decreased [OR: 0.72, 95% CI: 0.54-0.95]. For all hrHPV combinations, the addition of HPV 16 was associated with a higher risk of CIN2-3.

**Conclusions:** An increase in number of hrHPV types is associated with lower CIN2-3 risk. Further cohort studies with larger samples are needed to clarify this relationship. The available evidence suggests that HPV 16 genotype plays an important role in patients with high-grade cervical lesions and has a negative impact on the cervix in concurrent multiple HPV infections.

**Key words:** Human Papilloma Vırus; coinfection; cervical intraepithelial lesions

Ginekologia Polska 2020; 91, 6: 324–330

## INTRODUCTION

Cervical cancer is the second most common type of cancer among women worldwide and the leading cause of mortality in developing countries. Virtually all cases of cervical cancer are attributable to human papilloma virus (HPV) infection caused by high risk genotypes, with HPV 16 accounting for approximately 50%, HPV 18 for 20%, and HPV 31, 33, 45 and 52 a total of 19% of the cases [1, 2]. In assorted communities, the shares of these different types of HPV vary. As a consequence of the prevalence of HPV, many countries have added HPV genotyping to cervical cytology in the community-based cervical cancer screening programs.

Although screening and treatment management for high-risk HPV (hrHPV) genotypes have well-defined guidelines, the issue of follow-up and treatment strategy in the presence of concurrent multiple HPV genotypes is less established. Co-infections of HPV types are common and have been documented in several epidemiological studies [3–6]. However, the data on the co-infections is limited, and the clinical meaning of the condition and its effect on the risk of cervical cancer and precancerous cervical lesions is not clear.

In this study, patients who were referred to our clinic for colposcopies based on community-based cervical cancer screening program and who were found to have cervical intraepithelial lesion (CIN) 1 and CIN2-3 after cervical biopsy were included. We investigate how an increase in the number of the high-risk HPV genotypes affect CIN2-3 risk and evaluate the relationship of different genotype combinations with cervical epithelial lesions.

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## MATERIAL AND METHODS

Within the scope of community-based cervical cancer screening program; we applied colposcopic cervical biopsy (criteria were one of the following *a) HPV 16, 18 positive; b) Cervical cytology normal or abnormal, high-risk HPV positive; c) cervical cytology abnormal*) to 840 patients who underwent fluid-based cervical smear and HPV genotyping between June 2015 and 2017 and referred to our clinic — Bakirkoy Dr. Sadi Konuk Training and Research Hospital, Department of Obstetrics and Gynecology, for colposcopies. Approval was granted for the present study from the Local Ethics Committee.

All cervical cytology samples were obtained through liquid-based Pap tests (Thin Prep Pap Tests). The liquid-based Pap tests were then reported according to the 2001 Bethesda system. In order to identify HPV genotypes, we analyzed cervical specimens using Hybrid Capture 2 for HPV 16, 18 and the other hrHPV (16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66 and 68) types.

According to the cervical cytology results and HPV genotyping, colposcopies were performed on the patients based on the guidelines of the American Society for Colposcopy and Cervical Pathology (ASCCP). Histological specimens were taken using a colposcopy-guided biopsy. A random biopsy was performed at the squamo-columnar junction of each patient who displayed no lesions caused by the colposcopy. All colposcopic examinations were performed by two gynecological oncology specialist, and the biopsies and final histological excision results were reviewed by one or two experienced gynecological pathologists. For all patients, demographic characteristics and the results of biopsies taken during colposcopies were recorded.

A new classification system, LAST, was used to identify the cervical lesions [7]. In this system, cervical lesions were classified as either high-grade (CIN2 and CIN3) or low-grade (CIN1). Follow-ups and treatments of the patients were managed according to the 2012 ASCCP guidelines.

The present hrHPV genotypes of patients with positive cervical intraepithelial lesions were recorded. All specimens with two or more detected HPV genotypes were considered as concurrent HPV infections.

The primary outcome measure is CIN2-3 risk compared to CIN1 group. The impact of the increase in hrHPV types was calculated by estimating how an increase in the number of concurrent hrHPV types changed the odds ratio of CIN2-3 risk.

### Statistical analysis

Statistical Package for the Social Sciences, Version 23.0 (SPSS Inc., Chicago, IL) was used for the statistical analysis. Kolmogorov Smirnov and Shapiro–Wilks tests were conducted to check the distribution of the data. Levene's test was used to assess the homogeneity of variances. For comparison of demographic characteristics, Mann-Whitney U test and Pearson Chi-Square Test were performed as appropriate. Bivariate logistic regressions were conducted for assessing CIN2-3 risk. CIN1 group was accepted as the reference group. Due to the limited number of patients included in the study, the model was adjusted for only demographic characteristics having p-value lower than 10% in the logistic regression model. Parity and the number of hrHPV types were the only variables that satisfied these criteria. Pearson Chi-Square Test or Fisher's Exact Test were used to compare the distribution of hrHPV types among different types of cervical lesions. 2-tailed p-values below 5% were assumed to be statistically significant. Data were presented as Mean ± SD, Median [Minimum-Maximum] or number (percentage).

## RESULTS

Colposcopic cervical biopsy revealed no dysplasia in 591 patients. We found cervical intraepithelial lesions in 249 patients. Of these 249 patients, 76 (30.5%) had CIN2-3 and 173 (69.5%) had CIN1. The distribution of single and concurrent hrHPV infections due to various hrHPV genotypes is summarized in Figure 1.

As Table 1 makes clear, there was no significant difference between the CIN groups in terms of demographic characteristics (age, obstetric histories, smoking status, contraception methods and age of marriage).

Table 2 summarizes how carrying HPV16 and HPV18 and increasing the number of hrHPV types affected the CIN2-3 risk compared to CIN1.

The relationships between the number of concurrent hrHPV types and CIN2-3 risk is presented in Table 3. None of the relationships is statistically significant. In particular, having 4 or more concurrent hrHPV infections has very low odds ratio for CIN2-3 risk, but is not significant [OR: 0.13 (0.02–1.07), p-value: 0.058].

Finally, the distribution of hrHPV types among CIN1 and CIN2-3 groups were summarized and compared in Table 4. HPV51 is significantly more common in patients with CIN1 compared to CIN2-3 group.

## DISCUSSION

In this cross-sectional study, we evaluate the relationship between multiple concurrent HPV infections and precancerous cervical lesions. The number of hrHPV genotypes is significantly lower in CIN2-3 patients compared to CIN1 patients. Hence, a higher number of hrHPV types is associated with lower CIN2-3 risk. The relationship gets stronger with concurrent phylogenetically independent HPV genotypes and with HPV infection with four or more concurrent hrHPV genotypes, but both of these relationships are statistically insignificant arguably due to the small sample sizes of the

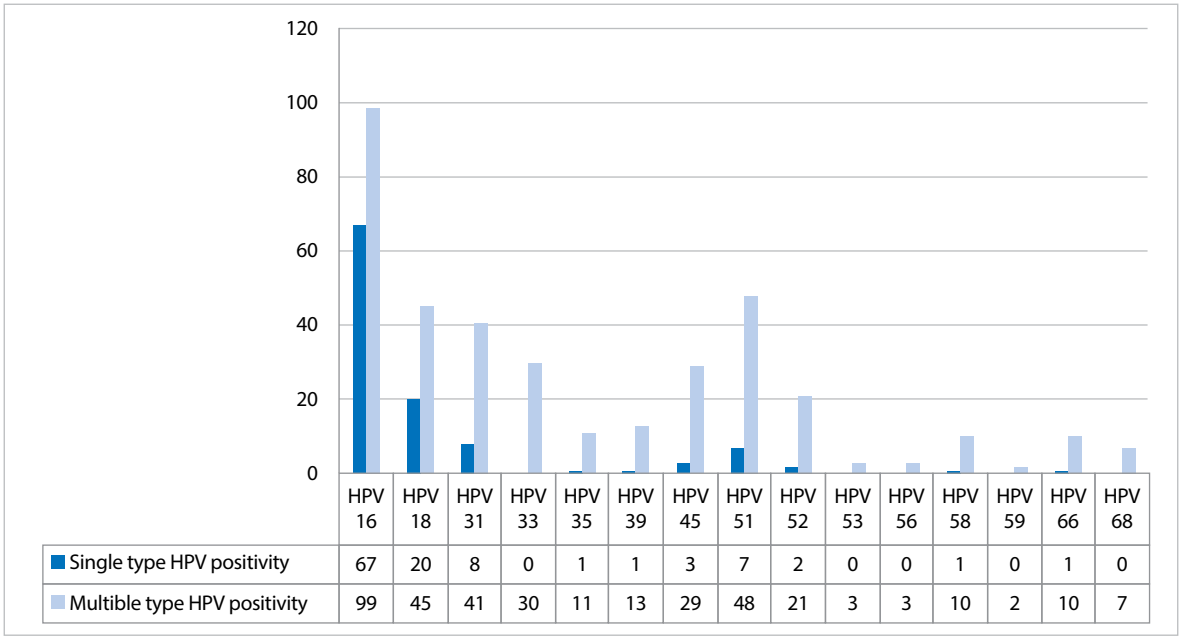


**Figure 1.** The distribution of single or multiple hrHPV infections related to various hrHPV genotypes ; HPV — Human Papilloma Virus

**Table 1. Demographic characteristics of cervical intraepithelial lesion groups**

| | CIN1 (n: 173) | CIN2-3 (n: 76) | p-value |
|---|---|---|---|
| Age ( mean ± SD) | 39.3 ± 6.6 | 39.2 ± 6.5 | 0.850[a] |
| | 39 [34; 44] | 39 [33; 44] | |
| Gravidy ( mean ± SD) | 3.2 ± 1.6 | 2.9 ± 1.4 | 0.352[a] |
| | 3 [2; 4] | 3 [2; 3.5] | |
| Parity( mean ± SD) | 2.4 ± 1.1 | 2.2 ± 1.2 | 0.164[a] |
| | 2 [2; 3] | 2 [2; 3] | |
| Education | | | 0.458[b] |
| No school | 15 (8.7%) | 7 (9.2%) | |
| Elementary school | 32 (18.5%) | 11 (14.5%) | |
| Middle school | 31 (17.9%) | 8 (10.5%) | |
| High school | 71 (41.0%) | 39 (51.3%) | |
| University | 24 (13.9%) | 11 (14.5%) | |
| Contraception | | | 0.750[b] |
| No contraception | 93 (53.8%) | 36 (47.4%) | |
| Oral contraceptives | 35 (20.2%) | 19 (25.0%) | |
| Preservatives | 24 (13.9%) | 10 (13.2%) | |
| Intrauterin devices | 21 (12.1%) | 11 (14.5%) | |
| Tubal ligation | 0 (0.0%) | 0 (0.0%) | |
| Smoker | | | 0,447[b] |
| No | 124 (71.7%) | 58 (76.3%) | |
| Yes | 49 (28.3%) | 18 (23.7%) | |
| Age of marriage (mean ± SD) | 22.6 ± 3.9 | 23.3 ± 4.6 | 0.401[a] |
| | 22 [20; 25] | 22 [20; 26] | |
| Number of HR-HPV genotypes | 2.1 ± 1.2 | 1.7 ± 0.8 | 0.047[a,*] |
| | 2 [1; 3] | 1 [1; 2] | |

CIN — cervical intraepithelial neoplasia; HR — high risk, HPV — Human Papilloma Virus;
Data are presented as mean ± SD; median (interquartile range) or number (percentage)
[a]Mann–Whitney U-Test; [b]Pearson Chi-Square Test; statistically significant comparisons are marked with*

**Table 2.** The relationship between different concurrent combinations of hrHPV genotypes and CIN2-3 risk in different samples

| CIN2-3 risk (reference group: CIN1) (OIITNO: One increase in the number of) | No. of women | OR (% 95 CI) | p-value | Adjusted OR (% 95 CI)[a] | p-value |
|---|---|---|---|---|---|
| In the total sample | | | | | |
| OIITNO any HR-HPV type | 249 | 0.72 (0.54–0.95) | 0.022* | 0.72 (0.54–0.96)[b] | 0.025* |
| adding HPV16 | 249 | 1.34 (0.74–2.40) | 0.331 | 1.02 (0.54–1.91)[c] | 0.963 |
| adding HPV18 | 249 | 0.75 (0.40–1.41) | 0.375 | 0.58 (0.30–1.14)[c] | 0.113 |
| adding HPV16 + HPV18 | 68d | 1.09 (0.38–3.14) | 0.879 | 0.42 (0.11–1.64)[c] | 0.213 |
| OIITNO α9 species different than HPV16 | 249 | 0.95 (0.64–1.43) | 0.813 | 1.02 (0.67–1.57)[c] | 0.912 |
| OIITNO α7 species different than HPV18 | 249 | 0.78 (0.42–1.43) | 0.415 | 0.79 (0.43–1.45)[c] | 0.442 |
| OIITNO HR-HPV types different than HPV16 and 18 | 249 | 0.71 (0.54–0.95) | 0.018* | 0.71 (0.53–0.95)[c] | 0.023* |
| Over the sample with HPV 16; | | | | | |
| OIITNO any HR-HPV type | 166 | 0.75 (0.54–1.03) | 0.079 | 0.76 (0.55–1.05)[c] | 0.092 |
| adding HPV18 | 166 | 0.97 (0.39–2.42) | 0.951 | 0.90 (0.35–2.28)[c] | 0.817 |
| OIITNO α9 species different than HPV16[d] | 141[e] | 1.15 (0.63–2.10) | 0.656 | 1.78 (0.88–3.60)[c] | 0.110 |
| OIITNO α7 species different than HPV18[e] | 141[e] | 0.69 (0.30–1.62) | 0.397 | 0.68 (0.29–1.59)[c] | 0.376 |
| OIITNO HR-HPV types different than HPV16 and 18 | 141[e] | 0.73 (0.50–1.06) | 0.096 | 0.75 (0.51–1.09)[b] | 0.126 |
| Over the sample with HPV18 | | | | | |
| OIITNO any HR-HPV type | 65 | 0.96 (0.60–1.55) | 0.875 | 0.92 (0.56–1.51)[c] | 0.748 |
| adding HPV16 | 65 | 1.62 (0.53–4.97) | 0.398 | 1.41 (0.44–4.51)[c] | 0.560 |
| OIITNO α9 species different than HPV16 | 40[f] | 1.24 (0.51–3.02) | 0.644 | 1.63 (0.55–4.81)[c] | 0.375 |
| OIITNO α7 species different than HPV18 | 40[f] | 0.98 (0.20–4.75) | 0.984 | 1.05 (0.20–5.36)[c] | 0.957 |
| OIITNO HR-HPV types different than HPV16 and 18 | 40[f] | 0.95 (0.49–1.87) | 0.888 | 0.94 (0.47–1.88)[b] | 0.856 |
| Over the sample with HPV16 and HPV18 | | | | | |
| OIITNO α9 species different than HPV16 | 25 | 0.78 (0.20–3.09) | 0.727 | 0.81 (0.19–3.56)[c] | 0.783 |
| OIITNO α7 species different than HPV18 | 25 | 0.67 (0.06–7.64) | 0.744 | 1.99 (0.07–57.19)[c] | 0.688 |
| OIITNO HR-HPV types different than HPV16 and 18 | 25 | 0.69 (0.26–1.83) | 0.453 | 0.68 (0.25–1.82)[b] | 0.444 |
| Over the sample without HPV16 and 18 | | | | | |
| OIITNO α9 species different than HPV16 | 43 | 0.54 (0.20–1.48) | 0.232 | 0.20 (0.04–1.11)[c] | 0.066 |
| OIITNO α7 species different than HPV18 | 43 | 0.92 (0.28–3.00) | 0.890 | 0.44 (0.07–2.90)[c] | 0.395 |
| Over the sample with only α9 species | | | | | |
| adding HPV 16 | 102 | 1.24 (0.40–3.90) | 0.713 | 1.23 (0.39–3.86)[c] | 0.728 |
| OIITNO α9 species different than HPV16 | 102 | 1.18 (0.61–2.29) | 0.629 | 1.47 (0.67–3.24)[c] | 0.342 |
| adding HPV 18 | 147 | 0.75 (0.35–1.60) | 0.448 | 0.95 (0.41–2.21)[c] | 0.896 |
| OIITNO α7 species different than HPV18 | 123 | 0.98 (0.45–2.12) | 0.954 | 0.98 (0.45–2.16)[c] | 0.968 |
| Over the sample with only α9 species different than HPV16 | | | | | |
| adding HPV18 | 44 | 0.60 (0.15–2.41) | 0.471 | 0.54 (0.06–4.86)[c] | 0.581 |
| Over the sample with only α7 species | | | | | |
| adding HPV 16 | 112 | 1.29 (0.53–3.18) | 0.578 | 1.33 (0.41–4.25)[c] | 0.636 |
| OIITNO α9 species different than HPV16 | 59 | 0.79 (0.37–1.70) | 0.542 | 0.81 (0.37–1.78)[c] | 0.596 |
| adding HPV 18 | 31 | 0.13 (0.02–0.89) | 0.038* | 0.07 (0.002–2.71)[c] | 0.156 |
| OIITNO α7 species different than HPV18 | 31 | 2.99 (0.70–12.68) | 0.139 | 0.63 (0.04–9.92)[c] | 0.743 |
| Over the sample with only α7 species different than HPV18 | | | | | |
| adding HPV16 | 76 | 0.25 (0.04–1.43) | 0.119 | 0.15 (0.02–1.41)[c] | 0.097 |

CIN – cervical intraepithelial neoplasia; HR – high risk; HPV — Human Papilloma Virus; OR — Odds ratio
[a]Odds ratios were adjusted for all demographic variables having effect on the CIN2-3 risk with p-value lower than 0.100
[b]The logistic regression model was adjusted for only parity according to[a]
[c]The logistic regression model was adjusted for parity and the number of HPV types except the investigated HPV types in each relevant analysis, according to[a]
[d]Women having only one of HPV 16 or HPV 18 excluded from the sample
[e]Patients with HPV 18 were excluded from the sample
[f]Patients with HPV 16 were excluded from the sample
Note: Statistically significant comparisons are marked with*. α9 species include HPV16, 31, 33, 35, 52 and 58. α7 species include HPV18, 39, 45, 59 and 68

**Table 3.** The relationship between the number of concurrent hrHPV types and CIN2-3 risk

| Number of HPV types | CIN1 (n: 173) | CIN2-3 (n: 76) | OR (% 95 CI) | p-value |
|---|---|---|---|---|
| | n (%) | n (%) | | |
| 1 | 72 (41.6) | 39 (51.3) | 1.00 (referent) | 0.611 |
| 2 | 50 (28.9) | 23 (30.3) | 0.85 (0.45–1.59) | |
| | | | | |
| 2 | 50 (28.9) | 23 (30.3) | 1.00 (referent) | 0.764 |
| 3 | 32 (18.5) | 13 (17.1) | 0.88 (0.39–1.99) | |
| | | | | |
| 3 | 32 (18.5) | 13 (17.1) | 1.00 (referent) | 0.058 |
| ≥ 4 | 19 (11.0) | 1 (1.3) | 0.13 (0.02–1.07) | |
| | | | | |
| Single | 72 (41.6) | 39 (51.3) | 1.00 (referent) | 0.157 |
| Multiple | 101 (58.4) | 37 (48.7) | 0.68 (0.39–1.16) | |

Data are presented as number (percentage)
*Reference group for calculating odds ratios are women with CIN1

**Table 4.** Comparison and distribution of hrHPV types for different types of cervical lesions

| | CIN1 (n: 173) | CIN2-3 (n: 76) | p-value |
|---|---|---|---|
| HPV16 | 112 (64.7%) | 54 (71.1%) | 0.331[a] |
| HPV18 | 48 (27.7%) | 17 (22.4%) | 0.374[a] |
| HPV31 | 30 (17.3%) | 19 (25.0%) | 0.162[a] |
| HPV33 | 24 (13.9%) | 6 (7.9%) | 0.182[a] |
| HPV35 | 8 (4.6%) | 4 (5.3%) | 0.760[b] |
| HPV39 | 13 (7.5%) | 1 (1.3%) | 0.070[b] |
| HPV45 | 24 (13.9%) | 8 (10.5%) | 0.467[a] |
| HPV51 | 50 (28.9%) | 5 (6.6%) | **0,009[a,*]** |
| HPV52 | 16 (9.2%) | 7 (9.2%) | 0.992[a] |
| HPV53 | 3 (1.7%) | 0 (0.0%) | 0.555[b] |
| HPV56 | 1 (0.6%) | 3 (3.9%) | 0.086[b] |
| HPV58 | 10 (5.8%) | 1 (1.3%) | 0.180[b] |
| HPV59 | 2 (1.2%) | 0 (0.0%) | 1.000[b] |
| HPV66 | 8 (4.6%) | 3 (3.9%) | 1.000[b] |
| HPV68 | 6 (3.5%) | 1 (1.3%) | 0.679[b] |

Data are presented as number (percentage)
[a]Pearson Chi-Square Test; [b]Fisher's Exact Test; statistically significant comparisons are marked with*; HPV — Human Papilloma Virus
Note: Patients might have more than one HPV genotype

subgroups. The evidence also suggests that the presence of an additional HPV 16 is associated with higher CIN2-3 risk for all different combinations of HPV genotypes.

HPV genotyping is part of the cervical cancer screening programs in many countries [8]. The epidemiological studies show that the prevalence of HPV types in each population is different [9]. Consequently, studies on HPV have limited generalizability except for the pathophysiologic interpretations. However, as most high-grade lesions are associated with HPV 16 and 18 universally, this relationship wastaken into account by diagnostic guidelines.

In this study, HPV 16 was found to be the most frequently genotype in both high-grade and low-grade lesions. In patients with high-grade lesions, HPV 31 and HPV 18 were found to be the second and third most frequent. For low-grade lesions, HPV 51 was the second most common (28.9%) and its frequency was significantly higher compared to patients with high-grade lesions (6.6%). Consistent with our findings, an epidemiologic study of over 1 million women in Turkey found HPV 51 to be the second most common type of HPV in non HPV 16,18 positive women [10]. It can also be conjectured that the HPV 51 genotype might be cleansed by the immune system during the progression of cervical intraepithelial lesion and therefore is not an important threat for precancerous lesions. These patterns should be taken into consideration when developing new and broader spectrum vaccines for immunization in the near future for the Turkish population.

There is a limited number of studies on concurrent HPV infections. A major reason for this was the difficulties associated with detecting other genotypes except HPV 16 and 18. Thanks to the polymerase chain reaction (PCR)-based assays developed especially in the last decade, the rate of detection of multiple HPV infections rose between 24.8% to 52.6% for different kits [11]. The improvements in detection in turn paved the way for investigating the impact of concurrent HPV genotypes on the risk of precancerous lesions and carcinoma of cervix.

Our findings suggest that increases in the number of hrHPV infection types decrease the frequency of CIN2-3. The mean number of hrHPV types are significantly higher in CIN 1 patients. The rate of multiple concurrent HPV infections was 48.7% in the CIN2-3 group and 58.4% in the CIN1 group. The 10% difference between the two groups suggests that some HPV genotypes are cleared by the immune system in the stage of progression of low-grade cervical lesions to high-grade lesion and the women with a lower number of hrHPV types might represent a sample with persistent HPV infection. The frequency of women with short term sexual intercourse is possibly higher in the low-grade lesion group.

The relationships between the number of hrHPV types and precancerous cervical lesions in various subpopulations are presented in Table 2. Arguably due to the small sample sizes, most of the relationships are statistically insignificant. An increase in α7 species of hrHPV types in the sample with α9 species lowers the risk of CIN2-3, probably because they are phylogenetically independent from each other. For all samples and hrHPV combinations, the addition of HPV 16 is associated with a higher risk of CIN2-3.

Because the present study is cross sectional, it does not allow establishing the causation between the number of hrHPV types and CIN2-3 risk. For this purpose, further clinical studies with cohort design are needed. The study, however, provides clues on the interactions between hrHPV types and CIN.

In the literature there are epidemiologic studies investigating multiple HPV infections and cervical carcinogenesis risk based on PAP-smear or colposcopy results. Salazar et al. reports that different combinations of HPV infections are associated with different risk ratios for high grade cervical lesions. The paper conjectures that intergenotypic competition or more effective immune response triggered by multiple infections might be decreasing the precancerous lesions' risk [12]. The limitation of the study was that the risks were not adjusted for demographic characteristics and the PAP-smear test used in study is underpowered to compare cervical lesions with pathological colposcopy evaluations. Dickson et al.'s study which was also based on PAP smear results found that multiple HPV type infections increase abnormal cytology risk relative to single type HPV infections [13]. Morrison et al. [14] and Chaturverdi et al. [3] find that LSIL (low-grade squamous intraepithelial lesion) risk increases substantially when HPV-16 and other HPV types are not present alone. Fife et al. [15] suggests that HPV types 51, 52, 56, and 58 might interact with HPV-16 to cause dysplasia or cancer. Our study also finds that the presence of HPV16 alongside with the other hrHPV types have larger odds ratios than 1 for CIN2-3. However, our other results seem to conflict with them and we can't generalize this situation for other hrHPV infections based on our subgroup analysis (Tab. 2).

Based on colposcopy results, Spinillo et al. in 2014 reports that multiple type HPV infections increases the risk of CIN3+ in both HPV 16 positive and negative women [16]. Recently, Debrot et al. conducted a follow-up study for assessing the CIN2-3 risk, but the study had small sample size and did not adjust for demographic characteristics [17].

The strengths of the present study are that it relies on colposcopy, and assesses the outcome based on the increase in the number of HPV infections while other studies mostly compare cervical lesions based on the presence of single or multiple infections. On the other hand, because the study is cross-sectional and does not track the persistence of HPV infections, it cannot establish causation. As for the sample size, it is large enough to assess the impact of the number of HPV types on CIN2-3 risk, but not large enough to conduct subgroup analysis without type 2 errors.

## CONCLUSIONS

All in all, the number of hrHPV types don't have a clear relationship with CIN2-3 risk. Some hrHPVs may increase while others may decrease the risk, and the specific combinations of the types appear to matter. Further cohort studies with larger samples are needed to establish these relationships in a clear way.

***IRB status:*** Bakirkoy Dr. Sadi Konuk Training and Research Hospital, Local Ethics Committee, Project no: 2018/410 Decision no: 2018-19-14

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VIA MEDICA

ORIGINAL PAPER / OBSTETRICS

Ginekologia Polska
2020, vol. 91, no. 6, 331–333

ISSN 0017–0011

DOI 10.5603/GP.a2020.0052

# Successful pregnancy in women with inferior vena cava stenosis — case report and discussion

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## ABSTRACT

**Objectives:** Inferior vena cava syndrome (IVCS) is a heterogenous group of symptoms resulting in obstruction of the main vein inflow . Common reasons are thrombotic changes and tumors. Incidence of inferior vena cava (IVC) anomalies is 0.3% in general population. Iatrogenic IVC lesions caused by catheter insertion play increasing role. Treatment varies depending on the condition.

**Material and methods:** 32-year old patient was diagnosed with IVC stenosis during infertility related preconception evaluation and informed about increased risk in planned pregnancy. Throughout the well progressing pregnancy patient received low molecular weight heparin.

**Results:** The diagnosis was confirmed intraoperatively during the planned cesarean section. Early postpartum period was normal and patient was discharged with antithrombotic prophylaxis.

**Conclusions:** Isolated IVC stenosis in pregnancy has not been yet reported in medical literature. Even though IVC anomalies may be associated with other congenital changes, in this case the central venous line treatment in infancy seems to be the most likely cause. Malformations are often accidentally diagnosed because patients are usually asymptomatic. CT and MRI are recommended diagnostic tools. Conservative treatment is recommended for asymptomatic patients, as opposed to surgical treatment for symptomatic. However, due to condition's rarity, there is no evidence based approach management.

**Key words:** inferior vena cava stenosis; inferior vena cava anomalies; varicose veins of parametrium; pregnancy complications

Ginekologia Polska 2020; 91, 6: 331–333

## INTRODUCTION

The inferior vena cava (IVC) is the largest vein in the body with a diameter of 18–32 mm. Inferior vena cava syndrome (IVCS) is a heterogeneous group of disorders causing obstruction of the inflow at various levels of the vein. It was well described by Sir William Osler as early as 1879 [1]. Development of IVC arises during the 4th to 8th week of gestation, and, due to its complexity, many malformations may appear [2]. Various IVC anomalies occur in approximately 0.3% of general population [3]. Sixty types of IVC malformations have been previously described [2], however, only few of them are clinically important. IVCS is often caused by thrombotic changes and tumors, and occurs rarely due to iatrogenic or genetic factors, aortic aneurysms, ascites, idiopathic retroperitoneal fibrosis, or retroperitoneal hematoma [4, 5]. Symptoms depend on the dynamics of the process and level of compression and may include edema of the lower extremities, varicose veins of the limbs, abdomen and chest, kidney failure, hepatomegaly, or pulmonary embolism. IVCS may appear in late pregnancy as a result of the enlarged gravid uterus compressing the IVC at supine position. This causes hypotension and may lead to stillbirth [6]. Treatment of IVCS varies depending on the primary condition. The authors present a rare case of an asymptomatic inferior vena cava stenosis and pelvic vein insufficiency during pregnancy which was found accidentally during infertility diagnosis.

## MATERIAL AND METHODS

A 32-year-old woman presented at a tertiary Outpatient Clinic for preconception consultation due to infertility. As

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part of her medical history, the patient reported having been treated in infancy for sepsis in a neonatal intensive care unit with the use of a catheter placed in the IVC from the femoral access.

Congenital thrombophilia was excluded during infertility evaluation. Transvaginal ultrasound revealed extensive varicose veins of the pelvis and the patient was referred to a vascular surgeon. The angio-MR examination showed stenosis of vena cava inferior in the infrarenal section with subsequent significant widening of the pelvic veins, especially in the right parametrium (Fig. 1 and 2). Such anomaly could have been a congenital hypoplasia or a post-thrombotic lesion. However, in this case the suspected cause was the central venous line treatment in infancy. The right ovarian vein was enlarged with a cross-section diameter of 18 × 16 mm. Widening of the paraspinal veins in the lumbar region was also present. This condition is very rare and evidence-based approach management has not been yet established. Endovascular correction such as angioplasty with stent implantation prior to pregnancy was considered despite the increased risk of thrombosis perinatally and cardiogenic shock with arterial hypotension during labor. Ultimately surgical treatment was not deemed necessary. It was recommended that the patient visits a perinatology specialist immediately after becoming pregnant. The patient was also informed about the increased risk of pregnancy.

Before and during her pregnancy the patient did not exhibit any clinical signs of IVCS such as visible collateral circulation, edema or varicose veins of lower extremities. As the stenosis was asymptomatic, a conservative approach to treatment was selected. Initially the patient received antithrombotic prophylaxis with a preventive dose of 40 mg of low molecular weight heparin (LMWH) and 75 mg of acetylsalicylic acid. During the third trimester her activated partial thromboplastin time shortened and the LMWH dose was increased to 80 mg.

At 38 weeks of the pregnancy the patient was complaining of severe, intensifying at night, itching. Pregnancy cholestasis was suspected so she was admitted to the Saint Sofia Hospital. The physical examination revealed small scars on the skin of the upper and lower extremities where vessels catheterization was performed in childhood. While the pregnancy cholestasis diagnosis was confirmed, no symptoms or signs of IVCS were observed.

## RESULTS

The patient was qualified for a cesarean section delivery due to ophthalmic reasons. A transverse, suprapubic, transperitoneal laparotomy was performed. The uterus was incised by the Cohen method. A healthy daughter of 3330 g and 55 cm was born, scoring 10 pts on the Apgar scale. Massive varicose veins of the parametrium were found intraoperatively. The vascular malformation of the vena cava

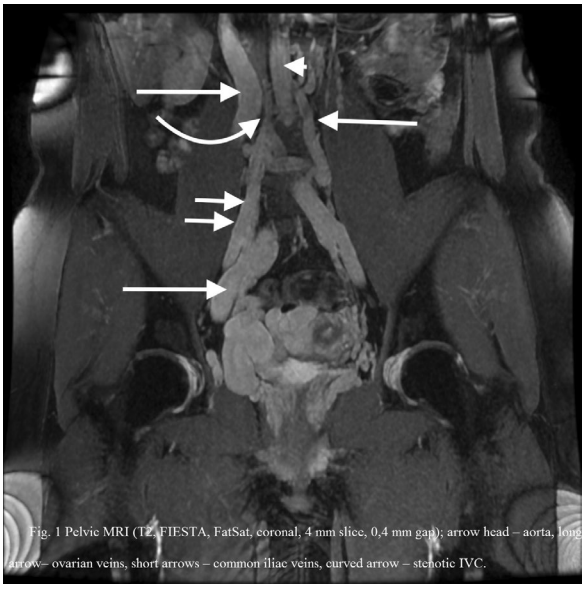


**Figure 1.** Pelvic MRI (T2, FIESTA, FatSat, coronal, 4 mm slice, 0.4 mm gap); arrowhead — aorta; long arrow — ovarian veins; short arrows — common iliac veins; curved arrow — stenotic IVC

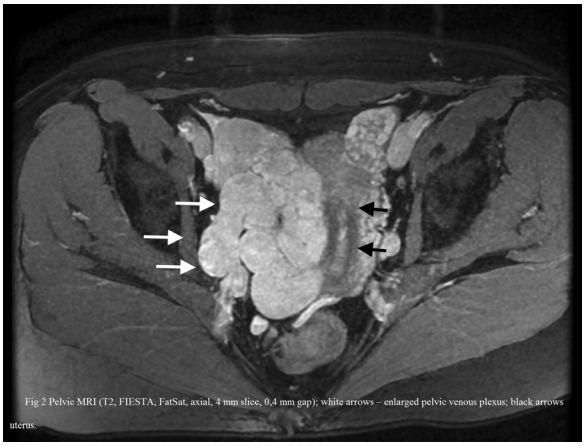


**Figure 2.** Pelvic MRI (T2, FIESTA, FatSat, axial, 4 mm slice, 0.4 mm gap); white arrows — enlarged pelvic venous plexus; black arrows uterus

was confirmed. The outside diameter of the malformed IVC was 10 mm width and its length being 5 cm.

The early postpartum period was normal. Anticoagulant treatment with 80 mg of LMWH was continued due to the thrombotic risk factors. The patient was discharged from the hospital on day 5 postpartum and antithrombotic prophylaxis was maintained for 6 weeks.

## DISCUSSION

Isolated IVC stenosis in pregnancy has not been yet reported in medical literature. The main causes of IVCS are deep vein thrombosis and tumors. Absence of the infrarenal segment of the inferior vena cava is a rare anomaly. Since 1957 only 16 cases confirmed by imaging examinations have

been reported in the medical literature [7–9]. Recent studies report that only 6% of IVC anomalies involved the renal or infrarenal segments [9]. Most of the congenital malformation are located suprarenal. Some authors suggest that the infrarenal absence of the inferior vena cava is embryonic in origin, others propose that it is the result of intrauterine or perinatal thrombosis [9, 10]. In our opinion iatrogenic stenosis following a catheter insertion plays an increasing role. IVC anomalies are often diagnosed accidentally as patients are usually asymptomatic. Patients with other thromboembolism risk factors such as pregnancy, especially with caesarean section delivery may develop a lower limb thrombosis and pulmonary embolism. In developed countries this is the main cause of death during pregnancy [9, 11]. The thrombotic risk is caused by pregnancy related hypercoagulability and stasis caused by the anomaly. The volume of the body fluids increases during pregnancy by up to 45%. That is why the risk of cardiogenic shock with arterial hypotension during labor in this case was considered. IVC anomalies may also occur as pelvic congestion syndrome with chronic pain. In such cases IVC stenting followed by classic coil embolization to ovarian veins might be viable option as the gonadal veins may be the main source of collateral flow back to the central venous system [12]. Inferior vena cava anomalies may be associated with other congenital anomalies, especially of the spleen and liver [8, 9]. Computer tomography and magnetic resonance imaging are the recommended tests in the IVCS diagnosis [9, 13].

## CONCLUSIONS

The management of IVC stenosis should depend on the condition and clinical symptoms. Conservative treatment is an option for asymptomatic patients. Thromboembolism risk can be managed with LMWH with the dose depending on all co-occurring rick factors. In symptomatic cases endovascular treatment is recommended [13]. A percutaneous balloon angioplasty with or without stent can be performed [12, 14, 15]. Evidence based approach management has not been yet developed as the condition is very rare [16]. Other thromboembolism risk factors should be excluded prior to conception and pregnancy in women with asymptomatic inferior vena cava stenosis should be carefully monitored. Antithrombotic prophylaxis should be implemented form the first trimester. Vaginal birth is recommended provided there are no other indications for cesarean section and immediate access to emergency treatment is ensured.

### *Conflict of interest*

The Authors declare no conflict of interest.

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VIA MEDICA

ORIGINAL PAPER / OBSTETRICS

Ginekologia Polska
2020, vol. 91, no. 6, 334–341

ISSN 0017–0011

DOI 10.5603/GP.2020.0058

# Expression of CD44 and IL-10 in normotensive and preeclamptic placental tissue

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## ABSTRACT

**Objectives:** We aimed to demonstrate cell-cell adhesion and apoptotic changes in preeclamptic placentas by examining the expression of CD44 and IL-10.

**Material and methods:** Placenta samples of 15 preeclamptic and 15 healthy 35–38th week-pregnant women were involved in the study. Tissue samples were taken only from the maternal side of the placenta and fixed in 10% formaldehyde, then blocked in paraffin wax and 5 µm-thick sections were cut and stained with Masson Trichrome. Antigen retrieval was performed for sections, incubated with CD44 antibody and anti-IL-10 antibody. After the application of streptavidin peroxidase followed by AEC chromogen solution, sections were counterstained with Mayer hematoxylin.

**Results:** In the preeclampsia group, increased CD44 positive expression was observed in maternal decidua cells and fibroblast cells close to root villi. CD44 was positively expressed in muscle cells around the blood vessels, mucosal connective tissue areas, syncytial nodes, and syncytial bridges. In the preeclampsia group, significant increased IL-10 expression was seen in subendothelial layers of the medium-sized vessels in the maternal region. IL-10 was also positively expressed in decidua cells outside the vessels, and inflamed connective tissue areas, chorionic villus cells with intense inflammation in intervillous spaces.

**Conclusions:** CD44 was found to be an essential molecule in the regulation of vascular permeability, inflammatory response, activation of the cells, cell-to-cell interaction, and the signaling pathways to which they are associated. Since IL-10 regulates appropriate pregnancy outcomes and contributes to the balance of anti-inflammatory signals via both paracrine and autocrine regulators of trophoblast activity, we proposed that it might be a key to elucidate the etiology of preeclampsia with CD44 receptor.

**Key words:** CD44; IL-10; normotensive and preeclampsia; placenta

Ginekologia Polska 2020; 91, 6: 334–341

## INTRODUCTION

Preeclampsia, a complication of pregnancy, occurs in 5–8% of pregnancies. Preeclampsia, which is among the hypertensive diseases of pregnancy, is one of the causes of maternal deaths all around the world [1]. Preeclampsia is diagnosed by onset of new hypertension and proteinuria after 20$^{th}$ week of gestation. İn the absence of proteinuria, preeclampsia can be diagnosed with hypertension accompanying by evidence of systemic disease (such as elevated of liver enzyme or thrombocytopenia) [2]. Preeclampsia is a risk factor for complications such as maternal renal failure, liver involvement, organ dysfunction, uteroplacental insufficiency, fetal growth retardation. Insufficiency of trophoblastic invasion of the placenta is important in the pathophysiology of preeclampsia. Changes in the spiral arteries are impaired in preeclampsia, cause placenta hypoxia, and decreased fetal blood circulation [3].

The cause of maternal clinical symptoms in preeclampsia is systemic endothelial dysfunction. Endothelium-dependent vascular tone control and vasoconstriction lead to hypertension; increased capillary permeability leads to loss of fluid into the third cavity, hemoconcentration and edema; increased glomerular permeability leads to proteinuria and coagulation mechanism leads to extensive intravascular coagulation. In preeclamptic patients, inadequate migration and invasion of cytotrophoblasts prevent formation of normal physiological changes in the spiral arteries, or they are confined to only decidua part of the spiral arteries [2, 4, 5].

Endothelial cells, trophoblasts and the immune cells in the endometrium during the implantation can synthesize

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many cytokines. A disorder in the cytokine environment may cause problems in the formation of placenta or may cause wide endothelial dysfunction, eventually leading to the development of preeclampsia [6].

Hyaluronic acid (HA) is a repeating non-sulfated glycosaminoglycan polymer of recurrent disaccharide units of N-acetyl glucosamine and p-gluconic acid. HA is an important component of extracellular matrix (ECM) of especially fast growing and remodeling tissues and plays a role in a variety of cellular functions including differentiation and inflammation [7].

CD44 mediates cell-to-cell and cell-to-matrix interactions through the hyaluronic acid (HA) receptor. It also plays an important role in cell adhesion and migration, tumor growth and progression via HA [8]. Apart from HA, CD44 has affinity and other ligands, such as osteopontin, collagen and matrix metalloproteinases (MMPs).

IL-10 is the main regulator of the inflammatory process and is present as a major immunomodulatory agent in the feto-maternal interface. The trophoblasts [9], decidua macrophages [10], natural killer (NK) cells [11] produce IL-10. It is most expressed at the feto-maternal interface of the extra villous cytotrophoblasts during the early pregnancy and villous cytotrophoblasts in late pregnancy [7]. Human leukocyte antigen (HLA-G) expression is inhibited lysis of maternal NK cells and induced by IL-10, which contributes to fetal allograft tolerance [12]. IL-10 also increases the resistance of trophoblasts to Fas-mediated apoptosis [13]. In our study, we aimed to demonstrate cell-cell adhesion and apoptotic changes in preeclamptic placentas.

## MATERIAL AND METHODS

The study was approved by the Medical Committee of Diyarbakir Maternity and Child Health Hospital. All protocols were approved by local ethics committee. All patients were informed and signed consent form. All placenta tissues were provided from the Diyarbakır Maternity and Child Health Hospital (Department of Obstetrics and Gynecology). The study included 15 pregnant patients with placenta previa and 15 healthy pregnant patients between gestational age. Preeclampsia criteria were defined by hypertension (systolic blood pressure ≥ 140 mmHg and/or diastolic blood pressure ≥ 90 mmHg) and proteinuria (> 300 mg in 24 h). Clinical blood tests were gathered and experimental biochemical analysis were performed on blood samples of patients. The placental tissues were immersed in 10% buffered formaldehyde. They were dehydrated in ascending alcohol series, cleaned in xylene and embedded in paraffin. Then 4 µm sections were cut and stained with Trichrom Masson.

### Immunohistochemical examination

All sections were bought to distilled water and for further immunohistochemical examination. Antigen retrieval process was performed in citrate buffer solution (pH: 6.0) for 10 minutes in a microwave oven at 700 W. Sections were permitted to cool down at room temperature for 30 minutes and washed in distilled water 2 × 5 minutes. 3% hydrogen peroxide ($H_2O_2$) was used for endogen peroxidase blocking for 10 minutes. Samples were rinsed in distilled water and washed in PBS. The sections were then incubated with mouse monoclonal anti-CD44 antibody (catalog no: sc-7297, Santa Cruz Biotechnology, Inc., Texas 75220 USA, 1: 100) and Mouse monoclonal anti-IL-10 antibody (catalog no: ab34843, Abcam, Cambridge, MA 02139-1517, USA,1:100) overnight at + 4°C. The next day, sections were cleaned with PBS and secondary antibody solution (Biotinylated Goat Anti-Mouse, Lab Vision) was applied for 14 minutes. Following PBS, streptavidin peroxidase solution (Streptavidin Peroxidase, Lab Vision) was performed for 15 minutes. Slides were washed 3 times in PBS and DAB chromogen solution were applied for 8 min. Sections were washed with distilled water and counter stained with 2 min Mayer hematoxylin. Slides were imaged with imager A2 Zeiss light microscope.

Semiquantitative scaling of sinsistial knot, congestion in blood vessels,fibrinoid acumulation nflamation and degeneration in decidua were carried out. The intensity of these changes were graded from 0 to 4 (0: no change, 1: low, 2: moderate, 3: intense 4;most intense). Semiquantitative scaling of immunoreactivity was carried out. The intensity of staining was graded from 0 to 4 (0: no staining, 1: faint staining, 2: moderate staining, 3: intense staining, 4;most intense staining).

### Statistical Analysis

Statistical calculations R version 3.2.3 (2015–12–10), Copyright (C) 2015 The R Foundation for Statistical Computing free software was used in the computer package program. Student t and Mann-Whitney U tests were used for statistical analysis. Statistical evaluations for $p < 0.05$ were considered significant.

## RESULTS

Independent Samples Test evaluation results, frequency values of categorical variables, Fisher's Exact Test calculations and statistical significance values were given in the tables below and statistical histopathological evaluation was evaluated according to these data. Histopathologic scoring and immunohistochemical expression p values are shown in the Table 1.

The characteristics of normotensive and preeclampsia patients are shown in Table 1. The placentas of the control and preeclampsia groups were compared histologically. CD44 and IL-10 expressions were also analyzed. Clinical results of normal pregnancy and preeclampsia pregnancy were compared (Fig. 1–4).

**Table 1.** Independent Sample T-test result of parameters

| Group Statistics | | | | | |
|---|---|---|---|---|---|
| **Groups** | | **N** | **Mean** | **Std. Error Mean** | **p value** |
| Age | Control | 15 | 28.20 | 1.72 | > 0.05 |
| | Preeclampsia | 15 | 31.40 | 2.74 | |
| Gravida | Control | 15 | 2.53 | 0.59 | > 0.05 |
| | Preeclampsia | 15 | 3.60 | 0.76 | |
| Parite | Control | 15 | 1.07 | 0.42 | > 0.05 |
| | Preeclampsia | 15 | 2.13 | 0.58 | |
| TAsist | Control | 15 | 113.00 | 2.65 | = 0.00 |
| | Preeclampsia | 15 | 154.20 | 4.32 | |
| TAdiast | Control | 15 | 71.53 | 1.75 | = 0.00 |
| | Preeclampsia | 15 | 96.33 | 1.45 | |
| Hemoglobin | Control | 15 | 12.19 | 0.40 | < 0.05 |
| | Preeclampsia | 15 | 10.95 | 0.44 | |
| Platelet | Control | 15 | 258.33 | 18.32 | > 0.05 |
| | Preeclampsia | 15 | 278.60 | 25.60 | |
| Glucose | Control | 15 | 78.87 | 2.73 | > 0.05 |
| | Preeclampsia | 15 | 75.54 | 3.31 | |
| Urea | Control | 15 | 16.06 | 1.04 | > 0.05 |
| | Preeclampsia | 15 | 18.28 | 1.60 | |
| Kreatinin | Control | 15 | 0.59 | 0.01 | > 0.05 |
| | Preeclampsia | 15 | 0.58 | 0.02 | |
| ALT | Control | 15 | 11.07 | 1.33 | > 0.05 |
| | Preeclampsia | 15 | 19.40 | 5.27 | |
| AST | Control | 15 | 22.07 | 2.98 | > 0.05 |
| | Preeclampsia | 15 | 27.07 | 6.90 | |
| Urine Protein | Control | 15 | 143.27 | 7.82 | = 0.00 |
| | Preeclampsia | 15 | 848.13 | 129.30 | |

Statistically $p < 0.05$ means, significantly difference between groups

### Histopathological Analysis

In placentas of control group, there were slightly enlarged medium-sized blood vessels large chorionic villi, regular syncytial cells with small fibrinoid structures and knots. The collagen fibers were organized in parallel and thin bands. Small villi structures in the intervillous area were normal (Fig. 4A). The chorionic villi of the preeclamptic placenta showed syncytial node, syncytial edema, and increased collagen fiber increase. In addition, heterochromatin appearance in syncytial bridges, degenerative changes in large nuclei, dilated and congested blood vessels and inflammation outside the vessels were observed (Fig. 4B).

High CD44 expression was observed in vascular basal membrane of cytotrophoblast and fibroblast cells in preeclampsia group. CD44 activity in the control group sections showed mild positive CD44 expression in the maternal decidua cells and fibroblast cells close to the root villi. Negative CD44 expression was observed in muscle cells around the blood vessels, mucous connective tissue areas, and syncytial cells (Fig. 5A).

Preeclampsia group: Increased CD44 positive expression was observed in maternal decidua cells and fibroblast cells close to root villi. CD44 was positively expressed in muscle cells around the blood vessels, mucosal connective tissue areas, syncytial nodes and syncytial bridges (Fig. 5B). Control group: IL-10 expression in trophoblast cells and connective tissue cells in floating villi was negative while it was positive in macrophage cells, syncytial nodes and bridges of root villi (Fig. 5C).

Preeclampsia group: Significant increased IL-10 expression was seen in sub endothelial layers of the medium-sized vessels in the maternal region. IL-10 was also positively expressed in decidua cells outside the vessels and inflamed connective tissue areas, chorionic villus cells with intense inflammation in intervillous spaces (Fig. 5D).

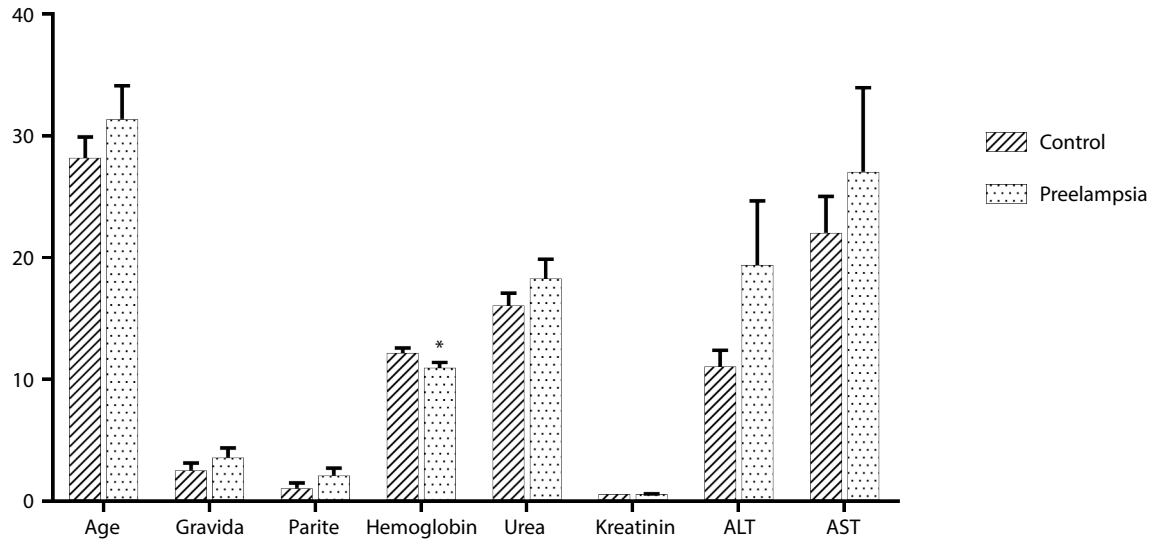


**Figure 1.** Independent samples T-test results of variables; Age, Gravia, Parite, Hemoglobin, Urea, Kreatinin, ALT and AST/ Different upper symbols of each column shows significantly difference between groups. (*$p < 0.05$)

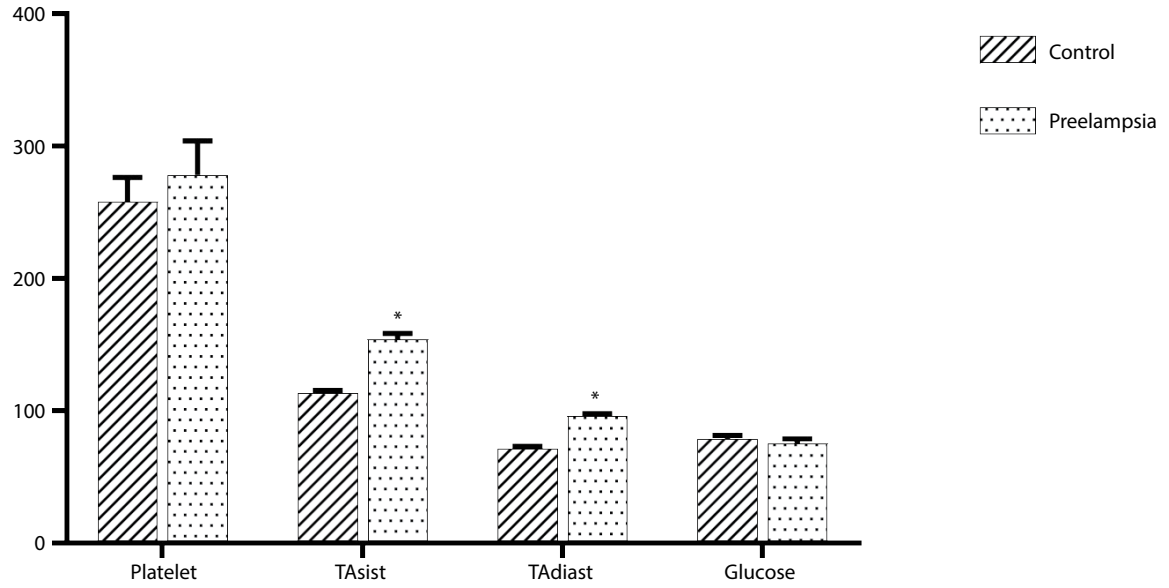


**Figure 2.** Independent samples T-test results of variables; Platelet, TAsist, TAdiast and glucose. Different upper symbols of each column shows significantly difference between groups. (*p = 0.00)

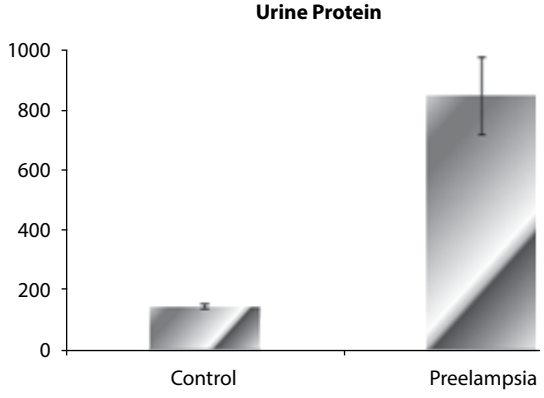


**Figure 3.** Independent samples T-test result of Urine. Different upper symbols of each column shows significantly difference between groups

## DISCUSSION

Preeclampsia (PE) is a pregnancy complication, which is the leading cause of maternal and fetal morbidity and mortality and occurs after 20 weeks of gestation. PE is a multisystem disease accompanied by hypertension and proteinuria (≥ 300 mg/24 h) or other symptoms (cerebral and vision symptoms, pulmonary edema, elevated serum creatinine levels, decreased platelet count [14, 15]. If systolic blood pressure is 160 mmHg or more and the diastolic blood pressure is 110 mmHg or more, it is defined as severe preeclampsia [15].

Hypoxia plays an important role in preeclampsia which is a pregnancy-specific syndrome characterized by maternal hypertension and proteinuria. Normally, the transformation of maternal spiral arteries occurs during placental development in the first weeks of pregnancy and this transformation is impaired in preeclampsia resulting in reduction of placental perfusion that triggers the inflammatory response. Starting by the second trimester of pregnancy, this inflammatory response elicits classic symptoms and findings in the diagnosis of preeclampsia. Systemic vasoconstriction and hypertension develop due to damage to the endothelial area of the vessels in the maternal circulation [16–19]. Hemolysis occurs because of systemic capillary endothelial damage, elevated liver enzymes and thrombocytopenia are together defined as HELLP Syndrome [17, 19]. Because of invasion of spiral trophoblast migration in PE, recurrent spiral arteries of the uterus may be impaired [20, 21].

Decidua arteriopathy results in a decrease in blood flow to the placental villi and distal villous hypoplasia. In the preeclampsia group of our study, chorionic villus syncytial node, syncytial edema, increased collagen fiber, heterochromatin appearance in syncytial bridges and degenerative changes in large nuclei were observed. In addition, dilatation and congestion in the blood vessels, inflammation around the vessels, showed the pathological condition resulting from hypoxia.

CD44 angiogenesis, resulting in endothelial cell proliferation, migration and differentiation, may contribute to synergistic stimulation. Immunohistochemical analysis has shown that endothelial cells, basement membrane and fibroblast cells express high CD44 levels. The role of CD44/hyaluronic acid interaction reported to be important for the differentiation of endothelial cells during angiogenesis [22]. Rui Zhu et al. [23] showed expression and function of hyaluronic acid/CD44 of

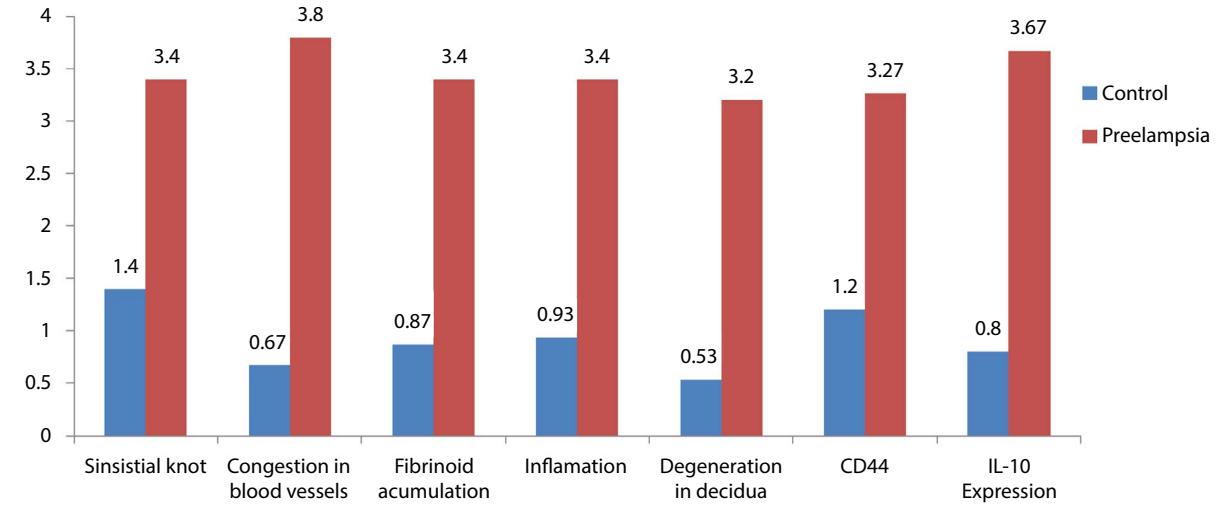


**Figure 4.** Graphical view of histopathological and immunohistochemical analyzes

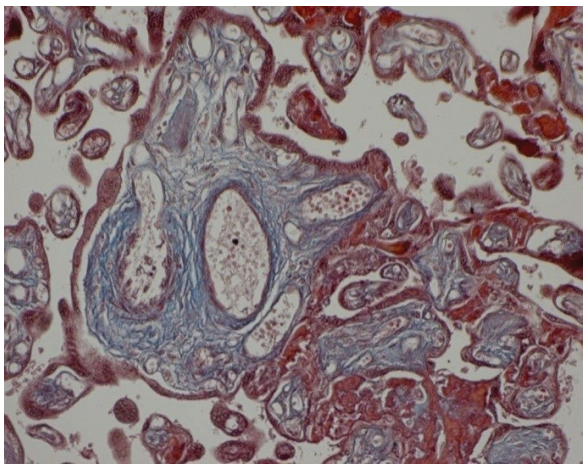

**Figure 4A.** Normotansive group:
Trichrom-Masson stainingX40
Immunohistochemical analysis

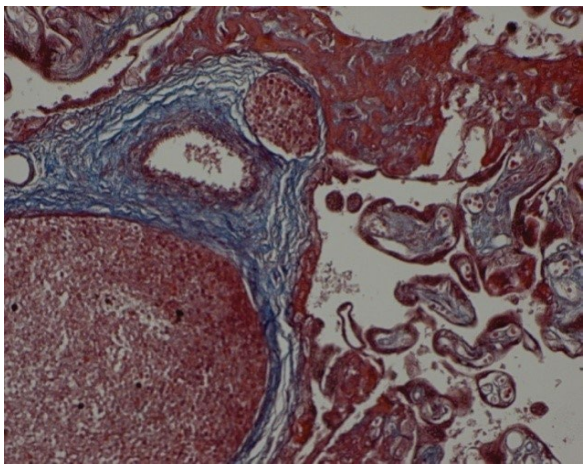

**Figure 4B.** Preeclampsia group
Trichrom-Masson stainingX40

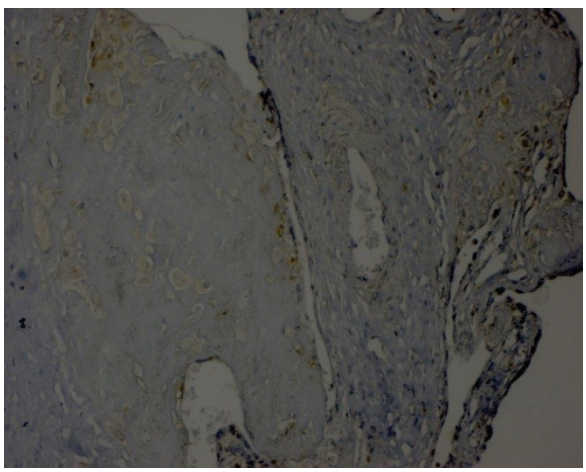

**Figure 5A.** Normotansive group:
CD44 immunstaining Bar 50µm

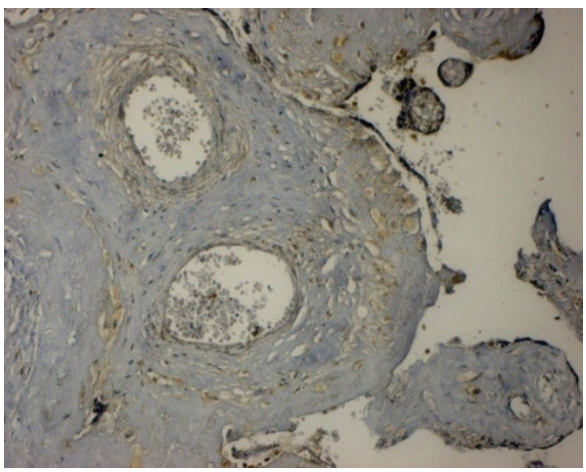

**Figure 5B.** Preeclampsia group
CD44 immunstaining Bar 50µm

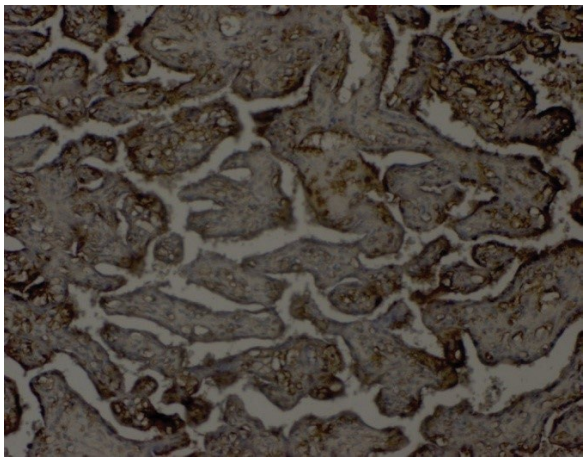

**Figure 5C.** Normotansive group:
IL-10 immunstaining Bar 50μm

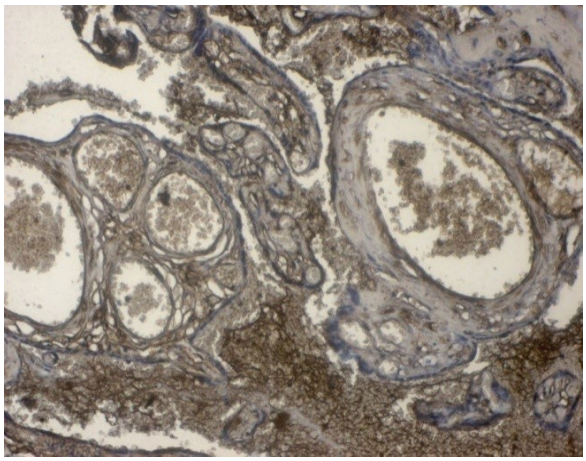

**Figure 5D.** Preeclampsia group
IL-10 immunstaining Bar 50μm

**Table 2. Histopathological scoring and immunohistochemistry expression results**

| Groups | | n | Mean | Std. Error | p value |
|---|---|---|---|---|---|
| Sinsistial knot | Control | 15 | 1.40 | 0.13 | = 0.000 |
| | Preeclampsia | 15 | 3.40 | 0.13 | |
| Congestion in blood vessels | Control | 15 | 0.67 | 0.48 | = 0.000 |
| | Preeclampsia | 15 | 3.80 | 0.41 | |
| Fibrinoid acumulation | Control | 15 | 0.87 | 0.51 | = 0.000 |
| | Preeclampsia | 15 | 3.40 | 0.63 | |
| Inflamation | Control | 15 | 0.93 | 0.59 | = 0.000 |
| | Preeclampsia | 15 | 3.40 | 0.63 | |
| Degeneration in decidua | Control | 15 | 0.53 | 0.51 | = 0.000 |
| | Preeclampsia | 15 | 3.20 | 0.56 | |
| CD44 | Control | 15 | 1.20 | 0.77 | = 0.000 |
| | Preeclampsia | 15 | 3.27 | 0.45 | |
| IL-10 Expression | Control | 15 | 0.80 | 0.56 | = 0.000 |
| | Preeclampsia | 15 | 3.67 | 0.61 | |

Mann-Whitney U test result of parameters; statistically $p < 0.05$ means, significantly difference between groups

human decidua stromal cells in the first trimester of pregnancy. They reported Hyaluronic acid (HA) stimulate proliferation and growth of decidua stromal cells by autocrine signaling, high levels of HA provide maintenance of normal pregnancy while low content and degradation of HA may cause early pregnancy loss.The presence of a functional CD44 molecule in the human placenta plays a role in the stabilization and orientation of the HA network, which is important in maintaining the placental structural integrity for CD44 [24]. CD44 is the main cell surface receptor for hyaluronic acid (HA), an integral component of the extracellular matrix and expressed in different cell types including epithelial, mesenchymal and hematopoietic cells, as well as immune cells. CD44 expression increases during inflammation and its variants of CD44v3 and CD44 v6 are also upregulated. CD44 was reported to participate in T cell activation, migration, and cell adhesion to HA during the immune response [25].

A study showed that in the presence or absence of placental pathology, hyaluronic acid was a key glycan in the regulation and distribution of other hyaluronic acids the organization and stabilization of extracellular matrix and glycocalyx, in the distribution of tissues, as well as HA fragments with different molecular weights interact with different types of CD44 receptors. HA negatively regulates the vascular permeability by activating signaling pathways associated with the formation of the cortical layer of actin microfilaments and intracellular contacts. It positively induces vascular permeability by triggering activation of the protease-activated receptor (PAR), thereby promoting the formation of actin stress fibrils and disruption of contact between cells [26, 27]. In our study, CD44 expression was observed in the maternal decidua cells, fibroblast cells close to the root villi, muscle cells around the blood vessels and mucosal connective tissue. In addition, the positive expression in the syncytial knots and bridges was thought to be important in altering the placental tissue structure of the secreting cells. Because HA regulates the systemic inflammatory response, different molecular weight hyaluronic acids have been reported to have various effects on pregnancy and may even promote pathology. According to the pathogenesis of PE, the main clinical manifestations of the disease are associated with insufficient localization, excessive systemic inflammatory response and endothelial dysfunction [28]. The role of cytokines in the pathogenesis of PE is important. Cytokines have many regulatory effects on interaction, uptake, activation, stimulation, killing and suppression of immune and non-immune cells. Cytokines

were reported to be involved in various events such as ovulation, implantation, placement and delivery during pregnancy. Cytokines such as TNFα stimulate the binding of CD44 expressed on peripheral monocytes to HA and CD44 expression, and adhesion to HA in hematopoietic cells is altered as a function of the cell activation stage [29].

Cytokines such as granulocyte-macrophage colony stimulating factor (GM-CSF), colony-stimulating factor-1, IL-3 and IL-10 contribute to the success of pregnancy [30]. In the first and second trimester placental tissues, IL-10 levels were moderate and higher in the third trimester period. Decidua cells, trophoblast cells and maternal uterine lymphocyte subgroups expresses IL-10 [31]. Abnormalities in IL-10 production may alter trophoblast invasion to uterus. It is proposed that Human Leukocyte Antigen-G (HLA-G), an antigen expressed by trophoblast, may regulate decidua cells expression and thus protecting maternal-fetal tolerance network. It is also emphasized that the role of inflammation is important for successful pregnancies. Chronic inflammation and lack of dissolution of anti-inflammatory cytokine producing cells can lead to various pregnancy disorders due to multiple factors. IL-4 and IL-10 play an important role in the successful pregnancy, and a deficiency of them was reported to contribute to infertility, spontaneous abortion and hypertensive pregnancy disorders [32]. The evaluation of placental tissue and serum samples from preeclamptic women showed that IL-10 production decreased. Endovascular interactions between trophoblasts and endothelial cells was reported to be impaired [33, 34]. In our study, there was a significant increased IL-10 expression in sub endothelial layers of medium-type blood vessel in the maternal region. Positive IL-10 expression was also found in inflamed connective tissue areas and decidua cells near the vessels. In addition, IL-10 activity increased in chorionic villus cells and intervillous spaces with intense inflammation.

## CONCLUSIONS

In conclusion, CD44 has been found to be an important molecule in regulation of vascular permeability, inflammatory response, and in activation of the cells and the cell-to-cell interaction and the signaling pathways to which they are associated. In a study, tumor necrosis factor (TNF)-α and interleukin (IL)-10 was studied as modulator on CD44 expression in Langerhans cells. They reported that 1) TNF-α significantly upregulates CD44 expression in a concentration-dependent manner and 2) IL-10 downregulates CD44 expression in a concentration-dependent manner. IL-10, a key regulator of immune system, not only has immunomodulatory activity, but also provides direct benefit to vasculature and promotes successful cellular interactions at the fetal-maternal interface. We thought that IL-10 act like TNF-α by upregulating CD44 expression. Thus, we speculate that IL-10 is important factor in blood cells and their activation by inducing HA to bind CD44.

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VIA MEDICA

REVIEW PAPER / GYNECOLOGY

Ginekologia Polska
2020, vol. 91, no. 6, 342–345

ISSN 0017–0011

DOI: 10.5603/GP.a2020.0054

# The use of levonorgestrel-releasing intrauterine devices in adolescents — own experience

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## ABSTRACT

It is estimated that 19% of adolescents in Poland begin their sexual life when they are 15 years old and more than 50% of people aged 17–19 years have already had their first sexual contact. World Health Organization recommends teenagers to use the method of 'dual protection' (condom and other method of contraception, *i.e.* intrauterine devices).

In this study, we sought to present our own experience in the field of the use of levonorgestrel-releasing intrauterine devices in adolescents and to compare it with the experience of other researchers worldwide.

Low- dose levonorgestrel-releasing intrauterine device is safe and effective method of contraception in adolescents. It is also an alternative treatment used in heavy chronic abnormal uterine bleeding. It can be also used as an alternative in women with cyanotic heart disease who have contraindications for standard contraception.

Gynecologists and pediatricians should be well informed in the topic of intrauterine device use among adolescents and they should provide them reliable knowledge in this field.

**Key words:** levonorgestrel-releasing intrauterine device; IUD; AUB; heavy menstrual bleeding

Ginekologia Polska 2020; 91, 6: 342–345

## INTRODUCTION

### Adolescent sexual behavior

It is estimated that 19% of adolescents in Poland begin their sexual life when they are 15 years old and more than 50% of people aged 17–19 years have already had their first sexual contact [1]. World Health Organization (WHO) estimates that 16 million adolescent girls get pregnant each year [2]. Although the access to medical information is getting easier, health-care worldwide is better than in the past and there is general technological progress, the number of teenage pregnancies is still growing. Unwanted pregnancy is connected with many consequences such as medical consequences of early pregnancy, sexually transmitted infections (STIs), unsafe abortions, social and personal consequences. Because of that, adolescent pregnancy prevention is one of the most important health-care issues of the twenty-first century.

European Society of Contraception and Reproductive Health Care emphasized that only 55% of young Europeans have access to education in the field of sexuality. Studies show that teenagers often don't know about the methods of contraception and risk sexual behaviors [3].

World Health Organization recommends teenagers to use the method of 'dual protection' (condom and other method of contraception) in order to simultaneously protect against pregnancy and STIs. Intrauterine devices (IUD) represent long-acting reversible contraception (LARC) and they are safe, effective and well-tolerated method of contraception for adolescents.

The Polish Society of Gynecologists and Obstetricians remains consistent with the recommendations of WHO. They emphasize that the method of contraception should be safe, effective, reversible, easy in use and easily accessible [4].

In this study, we sought to present our own experience in the field of the use of levonorgestrel-releasing intrauterine devices (LNG-IUDs) in adolescents and to compare it with the experience of other researchers worldwide.

## CURRENT STATE OF KNOWLEDGE AND OWN EXPERIENCE

### Levonorgestrel-releasing intrauterine devices in adolescents

Over 150 million women worldwide use the IUDs for contraception [5]. As a result of various contraceptive ac-

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tions, the efficacy rate of the LNG-IUD is high, which makes it a great option for young women.

Four hormonal levonorgestrel-releasing IUDs (LNG-IUDs) are currently available in Poland (containing different doses of LNG: 13.5 mg, 19.5 mg, 52 mg). IUDs should not be used in patients before the first menstruation. We should emphasize that only some devices are dedicated for teenagers [data about being used in women under 18 years old is contained in the Summary of Product Characteristics (SmPC)]. What is more, the devices have different dimensions. We recommend using low-dose IUDs that are fitted to a smaller uterus.

## Six — step procedure to introduce IUD

Physicians from INTRA (Intrauterine Contraception Translating Research Into Action) have proposed 7-minutes algorithm of introducing intrauterine device, which we highly recommend in everyday practice [6]:

Step 1

- Adjust patient's expectations to her needs. Evaluate experience and knowledge about various contraceptive methods.
- Ask simple questions: What contraceptive did you use? Are you currently sexually active? Are you planning a pregnancy?

Step 2

- Present long-acting reversible contraception (LARC).
- Regardless of the reason for the visit, ask the patient about her contraception.
- Assess the patient's knowledge and interest in intrauterine contraception.

Step 3

- Inform about the potential benefits of intrauterine contraception.
- When the patient is interested, expand your information to include: high efficacy, no need for daily, weekly or monthly administration, rapid reversibility, cost- effectiveness, and quick insertion in most women.

Step 4

- Confirm method reliability and dispel doubts.
- Inform about potential side effects such as the risk of ectopic pregnancy, perforation, expulsion or prolapse, infection, and changes in menstrual bleeding patterns.
- Refer to side effects for other methods of contraception and pregnancy itself.

Step 5

- Help to make a decision.
- Share your knowledge and clinical experience. Ask if the patient is interested in seeing the system. Be honest when you talk about discomfort issues.

Step 6

- Confirm the decision and plan the insertion of the IUD.
- Inform that if serious side effects occur, they should contact their physician immediately.

In addition, The Polish Society of Gynecologists and Obstetricians recommends performing before introducing a new method of contraception:

- Physical examination including blood pressure measure;
- Gynaecological examinations;
- Transvaginal ultrasound examinations;
- Breast examinations;
- Cytology test.

According to WHO recommendations, hormonal contraception can be introduced in teenagers [2]:

- 2 years after menarche;
- In the case of dysmenorrhea.

LNG-IUDs are also a great alternative for women with cyanotic heart disease with contraindication to standard hormonal therapy use.

## LNG-IUD as a method of treatment of abnormal uterine bleeding

Abnormal uterine bleeding (AUB) is one of the most frequent condition with which teenagers present in gynecologic office. The most common causes of AUB in adolescents are anovulation and coagulopathy. The initial management of AUB in adolescents is based on whether the AUB is acute or chronic. The second type occurs when uterine bleeding is abnormal in volume, regularity, and/or timing and has occurred for most of the past 6 months. It does not require urgent treatment (unlike acute AUB). LNG-IUD is an excellent management option for a patient experiencing chronic abnormal uterine bleeding. It is more effective than oral medication as a treatment for heavy menstrual bleeding and it is associated with a greater reduction in heavy menstrual bleeding and improvement in quality of life, and appears to be more acceptable long term [7].

In our practice, we have been using several LNG-IUD as an alternative method of treatment in heavy chronic AUB in adolescents (off-label treatment) without observing side effects. Our experience in this field involves 3 girls aged 14–16 with contraindications to use estrogens (implementation of heart valve in childhood, congenital cyanotic heart disease). All three patients have not begun their sexual life yet. Formal consent from their parents for implementation of LNG-IUD was required in all 3 cases.

We would like to present closer a case of 14-year-old girl with cyanotic heart disease who presented with abundant menstruations and irregular cycles. The girl underwent several cardiac surgeries and she is still prepared for subsequent interventions. She uses permanent anticoagulants — under INR control and she has absolute contraindications for estrogen use. On physical examination: peripheral cyanosis, „rod-like fingers". Gynecological

condition was suitable for age (Th3/P3/A3). Gynecological transabdominal ultrasound examination revealed an image suitable for age, without signs of pathology, endometrium of 5 mm. Hemoglobin level on first visit was 8 g%. The patient was diagnosed with severe AUB. Firstly, we tried using dydrogesterone for several months with no effect. Final therapeutic decision was made at the age of 15: we have inserted intrauterine hormonal contraceptive systems (LNG-releasing) under general anesthesia, after obtaining full formal parental consent. Patient's clinical state was re-evaluated after 1, 3 and 6 months. We have observed complete blockage of uterine bleeding with no side effects.

Our second patient was a 15 years old girl with Turner Syndrome (45 X0/46 XX), after 3 years of GH therapy with spontaneous menarche at the age of 11. In addition, she suffered from congenital heart disease (bipolar aortic valve — mild form, under clinical follow-up) and she had relative contraindications for estrogen use. The patient presented with very abundant menstruation, lasting up to 21 days and irregular menstrual cycles. Her gynecological condition was suitable for age (Th5/P5/A5). Gynecological transabdominal ultrasound examination revealed an image suitable for age, without signs of pathology, endometrium of 8mm. Hemoglobin level on first visit was 10 g%. The patient was diagnosed with moderate AUB. First stage therapy consisted of dydrogesterone 10mg twice a day between 16–25$^{th}$ cycle's days (insufficient effect). Our final therapeutic decision was inserting intrauterine hormonal contraceptive systems (LNG-releasing) at the age of 16.5 under general anesthesia, after obtaining full formal parental consent. Patient's clinical state was re-evaluated after 1, 3 and 6 months. We have observed complete blockage of uterine bleeding with no side effects.

### LNG-IUD in the treatment of endometriosis and chronic pelvic pain

Although LNG-IUD is not dedicated to be used as a first-line treatment of endometriosis and chronic pelvic pain (no registration in SmPC), we have experience in treating young patients with diagnosed endometriosis (off-label treatment). In 6 adolescents we have introduced low-dose LNG-IUD with success. Firstly, the girls were treated with standard methods, unfortunately without any effect. All six patients have not begun their sexual life yet. Formal consent from their parents for implementation of LNG-IUD was required in all 6 cases. Literature lack information about the use of LNG-IUD in adolescents suffering from endometriosis, so we should conduct further research in this topic.

Effects on the menstrual bleeding are expected in most women using the LNG-IUDs. Such changes are the result of a direct effect of levonorgestrel on the endometrium and may not be correlated with ovarian activity. Irregular bleeding and spotting often occur in the first months of use. Then, as a result of strong endometrial inhibition, the duration and reduction of menstrual bleeding are reduced. Minor bleeding often turns into rare bleeding or amenorrhea. In clinical trials, rare bleeding and/or amenorrhea developed gradually. In non-menstrual women, multiple pregnancy tests are not necessary unless there are other signs of pregnancy. If the bleeding gets worse and/or becomes more irregular over time, appropriate diagnostic measures should be taken because irregular bleeding may be a sign of endometrial polyps, hyperplasia or cancer. Heavy bleeding may be also a symptom of expulsion of the intrauterine therapeutic system. In most women, after the IUD is put on, the menstrual bleeding pattern changes. Over time, the percentage of women with amenorrhoea and with rare bleeding increases, while the percentage of women with prolonged, irregular and frequent bleeding decreases [8].

### Safety

Some physicians are concerned about using IUDs in young women because they are higher risk of STIs. However, despite concerns, studies show that insertion of IUD in young women (including teenagers) is not connected with higher risk of STI when compared to older women. The risk of pelvic inflammatory disease (PID) in the first 20 days after insertion is less than 1%, and then decreases to around 1 in 1000 [8]. However, the American Pediatric Society recommends screening for Chlamydia and Neisseria before inserting IUD due to the significant risk of transmission of infections in the adolescent group [9]. We recommend conducting a precise interview (*i.e.* information about many sexual partners, questions about risk factors of PID, the occurrence of PID in the past) with the patient before introducing the system [8]. In our practice, we have never observed PID in a teenage patient after inserting LNG-IUD.

## CONCLUSIONS

IUDs are considered to be safe and well tolerated method of contraception among adolescents. However, young women often have to face some barriers connected with IUD access (*i.e.* financial barriers). Gynecologists and pediatricians should be well informed in the topic of IUD use among adolescents and they should provide them reliable knowledge in this field. Teenagers often seek effective, safe and easy method of contraception, which does not require daily reminding. LNG-IUD should be always considered as a method of contraception in young women.

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VIA MEDICA

REVIEW PAPER / OBSTETRICS

Ginekologia Polska
2020, vol. 91, no. 6, 346–351

ISSN 0017–0011

DOI 10.5603/GP.2020.0064

# The usefulness of periostin determination in gynecology and obstetrics

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## ABSTRACT

Periostin (POSTN) is a multifunctional glycoprotein that belongs to the group of extracellular matrix (ECM) proteins. Due to the molecular structure, cellular interactions, tissue locations as well functions of POSTN, we realize that its pivotal role is organization and regulation of ECM microenvironment. In available databases there is a lack of data summarizing current knowledge about POSTN expression in the field of gynecology and obstetrics. We conducted a search in PubMed of the National Library of Medicine and Google Scholar. Databases were extensively searched for all original and review articles/book chapters published in English until December 2019 and related to periostin expression. All relevant articles were reviewed and presented as appropriate.

In the field of POSTN expression there is only one paper evaluating its involvement in cervical cancer cell metabolism and only two studies analyzing its myometrial commitment: maintenance during pregnancy and induction of parturition in physiology as well control of fibroids biology in pathology. Much more attention has been devoted to the expression of described protein in the endometriosis, and above all in ovarian cancer. Finally, a few studies carried out among pregnant women were presented.

In this review study we presented current knowledge about periostin expression in the field of gynecology and obstetrics. Many achieved results are interesting and further studies are needed to verify some hypotheses. Structure, signaling pathways as well many functions of periostin are well-described. However, as it was clearly shown there is a lot of unknown issues which are waiting to be explored.

**Key words:** periostin; POSTN; cancer; neoplasm; endometriosis; myoma; leiomyoma; fibroid; gestation; pregnancy; miscarriage

Ginekologia Polska 2020; 91, 6: 346–351

## INTRODUCTION

Periostin (POSTN) also called osteoblast-specific factor 2 (OSF-2) is a multifunctional glycoprotein that belongs to the group of extracellular matrix (ECM) proteins. Physiological POSTN expression was disclosed in wide variety of normal fetal and adult tissue; *i.a.* embryonic periosteum, periodontal ligament, placenta, cardiac valves, adrenal glands, lung, thyroid, stomach, colon, vagina, ovary, testis, prostate, and breast. It has been shown to be an important regulator of bone and tooth formation and maintenance, and cardiac development and healing [1, 2]. Additionally, it was also found that POSTN is expressed in tissues under stress conditions, such as pressure or volume overload in heart, skeletal muscle after injury or cellular response to hypoxia. Moreover, POSTN overexpression has been reported for some pathological states, *i.e.* chronic sinusitis, allergic airway inflammation as well in many cancer types [1–3].

The human POSTN is encoded by gene located on chromosome 13q13.3, and has two isoforms (779 and 836-amino acid protein with a molecular weight of 87 kDa and 93.3 kDa, respectively) identified through studies on human placenta and osteosarcoma cDNA libraries [2]. POSTN has an N-terminal signal peptide, a cysteine-rich region, four internal homology domains and a carboxyl terminal region. There is a typical signal sequence at the N-terminus suggesting that it has the potential to be a secreted protein. The C-terminus is hydrophilic and can undergo alternative splicing at the transcriptional level to form splice variants or

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isomers of POSTN. It has eight types of homologous isoforms in human tissues that are associated with the occurrence of specific tumors [1, 3].

POSTN by interaction with the integrins ($\alpha_v\beta_3$, $\alpha_v\beta_5$, and $\alpha_6\beta_4$) activates the Ilk–PI3K/Akt and focal adhesion kinase (FAK)-mediated signaling pathways. It is responsible for ECM remodeling, as it controls adhesion processes and biomechanical properties of connective tissue (*i.a.* regulation of collagen fibriollogenesis) [4, 5]. It creates an environment for angiogenesis and lymphangiogenesis, independent proliferation, avoidance of apoptosis and the ability for cells to re-enter the cell cycle, which in pathology leads to promotion of cancer cell survival, invasion and metastases, mostly on basis of epithelial-mesenchymal transition (EMT) phenomenon [5, 6].

Due to the molecular structure, cellular interactions, tissue locations as well functions of POSTN we realize that its pivotal role is organization and regulation of ECM microenvironment. In available databases there is lack of data summarizing current knowledge about POSTN expression in the field of gynecology and obstetrics.

## Methodology of Data Obtaining and Analysis

We conducted a search in PubMed of the National Library of Medicine and Google Scholar. Databases were extensively searched for all original and review articles/book chapters published in English until December 2019 and related to periostin expression using the following keywords (one or in combinations): periostin, POSTN, gynecology, cancer, neoplasm, endometriosis, myoma, leiomyoma, fibroid, obstetrics, gestation, pregnancy, miscarriage. Moreover, additional articles from the reference sections of the reviewed articles were searched. Overall, all relevant articles were reviewed and presented as appropriate.

## Cervix

In the field of cervical expression of POSTN there is only one paper evaluating its involvement in cancer cell metabolism. Han et al. investigated human cervical cancer samples as well commercial cell lines (HeLa and SiHa). They disclosed overexpression of POSTN in studied cases. Moreover, they observed that POSTN knockdown in tested cell lines significantly decreased cell viability, migration and invasion, and reduced EMT. Furthermore, POSTN knockdown suppressed the activation of Akt/mTOR signaling pathway. In well-designed experiment Han et al. [7] demonstrated that of MALAT-1/microRNA-202-3/POSTN axis regulates cell viability, migration and invasion as well EMT of cancer cells *via* Akt/mTOR signaling pathway. Further research would be greatly beneficial, especially due to unknown prognostic and predictive value of this protein in cervical cancer.

## Endometrium

The endometrium is composed by two types of cells *i.e.* endometrial stromal cells (ESC) and endometrial epithelial cells (EEC) which both may have eutopic (physiological) and ectopic (pathological) localization. First study on POSTN expression in eutopic endometrium was conducted by Hiroi et al. [8] in 2008. A significant increase in *POSTN* expression was observed during mid-proliferative and early secretory phases while during late proliferative, mid-secretory and late-secretory phases were decreased. Observations strongly correlated with estrogen and progesterone supplementation during the study. Moreover, they disclosed increased immunohistochemical (IHC) POSTN expression in ESC during early proliferative, mid-proliferative and early secretory phases as well in EEC in late secretory phase. The authors clearly showed that POSTN expression is controlled by ovarian steroid hormones, which may have strong impact on physiological pregnancy and pathological processes *e.g.* endometriosis.

Endometriosis is a chronic inflammatory disease caused by ectopic endometrium manifesting itself by recurrent pain and infertility in women of reproductive age. Repeated tissue injury, repair and subsequent fibrosis are its crucial elements. Microscopic image is characteristic and well-described, however, due to lack of known etiology as well signaling pathways, further studies are needed. Therefore, Shen et al. [9] investigated IHC POSTN expression in eutopic and ectopic endometrium of women diagnosed with endometriosis. They observed cyclic variation of eutopic stromal IHC POSTN expression; stronger in the proliferative than in the secretory, similarly to Hiroi's observations. Moreover, Shen et al. found the strongest POSTN expression in ectopic endometrium, which suggests involvement of POSTN in the pathophysiology of endometriosis. In 2015 Xu et al. [10] studied mRNA and protein expression of POSTN in ectopic ESC. They disclosed that POSTN enhanced ESC migration, invasion as well adhesion due to the Ilk/Akt signaling pathway. Moreover, they speculated that due to the known way of action, POSTN may be a new therapeutic target in endometriosis. Based on the above data, Zheng et al. [11] decided to verify POSTN expression in EEC and check its influence on EMT. They received results concordant with those by Xu et al., *i.e.* POSTN enhanced invasion and migration abilities of EEC as well as, facilitated the EMT through Ilk-Akt signaling pathway. Further studies conducted in 2018 by Logan et al. [12], investigated molecular pattern of EEC and ESC of eutopic endometrium women with endometriosis. They performed fluorescence-activated cell sorting for paired sibling RNA sequencing and miRNA microarray and disclosed 151 and 215 differently expressed genes as well 9 and 16 differently expressed miRNAs in ESC and EEC, respectively. POSTN was found to be downregulated in ESC.

Results of this study may be crucial in perspective of eutopic endometrium biology in patients affected by endometriosis and their pregnancy expectations.

Other studies on POSTN expression in endometriosis are focused on identification therapeutic approach. Ganieva et al. determined whether transcription factor 21 (TCF21) is involved in the development of endometriosis as an upstream regulatory gene of POSTN [13]. They separately verified expression of POSTN and TCF21 in different types of endometriosis. They disclosed that expression of POSTN and TCF21 was absent in normal endometrium of women without endometriosis, weakly positive in eutopic endometrium of women with endometriosis, moderately positive in ovarian endometriosis, and strongly positive in deep infiltrating endometriosis. Additionally, usage of siRNA against human TCF21 suppressed POSTN expression, as well transfection of TCF21 plasmid vector into stromal cells of women without endometriosis, which natively expressed neither POSTN nor TCF21, resulted in presence of their expression. In view of the above information, Ganieva et al. suggest that TCF21 may be promising therapeutic target in endometriosis.

## Myometrium

The myometrial expression of POSTN is described only in two studies. Liu et al. [14] analyzed molecular mechanism responsible for balance between maintenance of pregnancy and induction of parturition. They compared contracted lower uterine segment (LUS) with relaxed uterine fundal myometrium (FUN) to assess transcriptome by RNA-sequencing, then RT-PCR and immunoblotting to validate sequencing results. Finally, cell contraction/adhesion assays and gene microarrays were used to study the cellular functions of identified genes. They disclosed higher levels of *HoxA13*, *PTGIS*, and *POSTN* in LUS at term prior to labor. The authors stated that regionalization of myometrial function may be mediated by *HoxA13*. Liu et al. [15] disclosed myometrial function of POSTN in physiology, however it was suspected to have its role in pathology. Jamaluddin et al. performed proteomic profiling of uterine fibroids to analyze ECM protein expression pattern. Using genetic sequencing and isobaric tagged-based quantitative mass spectrometry (iTRAQ) they analyzed samples in two main groups: MED12 positive and negative mutation (one of the most common anomalies in fibroids). They disclosed sets of down and upregulated proteins, however regardless MED12 status POSTN was significantly upregulated. Performed western blotting (WB) and IHC analyses confirmed proteomic observations. They finally concluded that increased POSTN is a hallmark of uterine fibroids regardless MED12 status and further studies on up-regulated ECM proteins should be performed.

## Ovary

Polycystic ovary syndrome (PCOS) is often diagnosed a metabolic disease associated with hormonal imbalance. There is growing evidence for links between POSTN expression and ovarian function. Chen et al. [16] evaluated by ELISA POSTN levels and compared it with parameters of triglyceride metabolism, chronic inflammation, and insulin resistance in PCOS patients. Serum POSTN levels in PCOS patients were almost 10 times higher than in controls. They discovered also that POSTN positively correlates with body mass index (BMI), uric acid, homeostasis model assessment of insulin resistance (HOMA-IR), high-sensitive C reactive protein (hs-CRP), and negatively with insulin sensitivity index (ISI). Chen et al. finally concluded that elevated levels of POSTN are associated with PCOS. In perspective of incidence of PCOS, presented results and lack of studies, further analysis is essential.

Tumor-specific molecular modifications have their place in the management of oncologic patients. POSTN is one of the glycoproteins identified to have it role in ovarian cancer (OC) [17]. One of the first studies showing overexpression of POSTN in OC was presented Gillan et al. in 2002. They concluded that POSTN can increase the motility of the cancer cells and their adhesion to the peritoneum *via* integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$. It results in detection of POSTN in the ascites of OC patients [18]. In the following studies POSTN properties were evaluated. Zhu et al. tested POSTN expression by cDNA microarray, northern blotting and IHC analyses [19]. Subsequently, using retroviral transfection, supported by WB and ELISA confirmation, they achieved POSTN expression in OC cells lines: OVCAR-3 and OV2008. The clinical effects of exogenous POSTN expression were assessed in orthotopic mouse models. Zhu et al. [19] found that POSTN overexpression did not change cell growth rates *in vitro*; however, it significantly promoted intraperitoneal tumor metastatic growth in immunodeficient mice, which was associated with increased tumor angiogenesis and decreased tumor cell apoptosis. It stays in line with the study by Choi et al. [20], who analyzed association between POSTN expression and lysophosphatidic acid (LPA), bioactive lipid crucial for the initiation and progression of OC. Treatment of SKOV-3 and OVCAR-3 cell lines with LPA induced expression of POSTN. Additionally, induced POSTN expression was abrogated by silencing of the LPA receptor expression using shRNA lentivirus. Recombinant POSTN stimulated adhesion and invasion of SKOV-3 cells what suggests that LPA may be associated with POSTN expression in the OC.

Subsequently, Abbot et al. performed glycotranscriptome comparative analysis of tissue and serum patients with OC to identify new specific markers. Selected transcripts of restricted glycosyltransferases were verified by lectins,

which showed tumor-specific glycosylation changes. The authors stated that regarding tested glycoprotein markers, thrombospondin and POSTN were overexpressed in OC and they can be potentially used to distinguish OC patient serum from normal serum [21]. Furthermore, proteomic study conducted by Tian et al. was aimed on identification glycoproteins useful in proper differentiation of histological subtypes of OC. The authors utilizing iTRAQ and WB disclosed that POSTN is overexpressed in most subtypes of OC and is unsuitable for differentiation. However, Tian et al. [22] simultaneously pointed its potential involvement in an early stages of OC formation, what may also constitute promising therapeutic target.

There are hypotheses that POSTN cleavage by blocking antibody or induced transcriptional gene silencing may constitute a highly effective method of treatment. Zhu et al. [23] developed neutralizing monoclonal antibody against POSTN called MZ-1. They performed their study on OC mice model derived from cell line A2780 to verify impact of MZ-1 on tumor growth and metastasis. The Authors believed that observed inhibited peritoneal metastasis after treatment with MZ-1 was result of specific inhibition of anchorage-independent growth and survival of POSTN expressing cells as well neutralizing effect of POSTN induced cancer cell migration and invasion. Moreover, *in vivo* administration of MZ-1 resulted in reduction in the number of metastasis.

In the subsequent studies prognostic and predictive value of POSTN was verified. Karlan et al. [24] performed RNA expression microarrays and Rosetta Similarity Search Tool to define sets of genes with potential relevance in OC, which finally were identified by array comparative genomic hybridization. They pointed out that high-grade serous OC patients with POSTN/TGFBI expression have significantly shorter overall survival (OS) being potential prognostic and therapeutic target. Ryner et al. [25] performed gene expression profiling on a discovery set of OCs with clinically well-defined response to chemotherapy as well as on an independent validation dataset of OC patients from the chemo treatment arm of the ICON7 trial. They defined "reactive stroma" gene signature that is specifically associated with primary chemo resistant tumors. IHC and RNA *in situ* hybridization analyses confirmed *POSTN*, *LOX*, and *FAP* genes to be associated with the clinical chemo resistance. Moreover, treatment with recombinant POSTN promoted ES-2 cell to carboplatin and paclitaxel treatment *in vitro*. Finally, they demonstrated that a high POSTN expression level predicts shorter progression-free survival (PFS) following first-line chemotherapy. In perspective of above observations and own study, Lister et al. found that promoter-directed small antisense non-coding RNAs can induce transcriptional gene silencing of POSTN what may result in a loss of cellular motility. Moreover, they observed that cell motility and possibly metastasis can be controlled by transcriptional and epigenetic regulation of POSTN [6].

In 2015 Tan et al. [26] presented transcriptomics database of human OC, referred to as CSIOVDB which comprises 3,431 microarray samples from 48 cohorts of private, in-house and public human OC datasets. They observed that elevated *POSTN* expression was characteristic for serous OC vs other histotypes, stage II-IV vs I, grade 2–3 vs 1 as well tumor resistant or refractory to first line chemotherapy vs sensitive ones. Moreover, Tan et al. [26] pointed that POSTN levels were significantly associated with OS, DFS as well showed that it was an independent predictor of PFS. Similarly, Sung et al. [27] analyzed microarray datasets and therefore performed *in vitro* study with A2780 cell line. They observed that POSTN treatment induced cisplatin resistance and activate Akt pathway, while its inhibition by selective inhibitor, MK-2206 abolished POSTN-induced Akt activation and cisplatin resistance *in vitro*. Overall, they showed that overexpression of stromal POSTN has independent poor prognostic value (OS and DFS) and is associated with higher percentage of platinum resistance. In 2018 Tang et al. [28] disclosed high POSTN levels in ascites OC patients (the same as Gillan et al. [18]) which correlated with level of CD163 tumor-associated macrophages. The high POSTN level and macrophage infiltration were inversely associated with relapse-free survival for OC patients. Additionally, with techniques described by Zhu and Lister, siRNA of POSTN and POSTN neutralizing antibody treatment showed that OC cell derived POSTN promoted the recruitment of macrophages and modulated their cytokine secretion profile [28].

In most up-to date studies, in 2019, Lu et al. [29] generated biotinylated form of human scFv antibody that targets the bisected N-glycans. They validated results in *in vitro* and *in vivo* studies indicating that obtained antibody may be useful for development diagnostic and therapeutic approaches for cancers expressing POSTN. Moreover, Sterzyńska et al. [30] investigated POSTN mRNA by RT-PCR and protein expression in cell lysates and cell culture medium by WB as well in cell lines by IF regarding development of chemotherapeutic resistance. They disclosed increased expression of POSTN in drug-resistant cells and stated that POSTN expression might be associated with the development of doxorubicin and methotrexate resistance in the primary serous OC cell line. Finally, in 2020 Kujawa et al. [31] analyzed expression of POSTN and fibronectin. They disclosed that higher expressions of both proteins were associated with shorter OS and demonstrated that combined score of fibronectins and POSTN was an independent prognostic factor for OS. What is new, Kujawa et al. [31] stated that elevated expression of fibronectin and POSTN was more common in fallopian cancers than in OC.

### Pregnancy

It has been shown that molecular processes occurring in the cancer cell metastasis are like those observed during human implantation. In view of above, ECM proteins responsible for cell adhesion allowing cancer cell maintenance may also have an impact on implantation. Therefore, Morelli et al. [32] analyzed tissue and serum expression of POSTN during pregnancy. They analyzed mRNA and protein expression in decidual and trophoblastic tissue as well serum protein level in between group of pregnant with spontaneous pregnancy loss and planned pregnancy termination. In all analyzed aspects POSTN expression was elevated in spontaneous pregnancy loss. The Authors speculated that POSTN may be a serum marker of good endometrial receptivity, embryo quality and predictive for pregnancy evolution. In the other study Freis et al. [33] tested by ELISA levels of POSTN in patients who conceived by IVF/ICSI and ovarian stimulation and with known first trimester outcome. They disclosed that increased levels of POSTN where observed in early pregnancy in patients with following miscarriage in contrast to patients with ongoing pregnancy who demonstrated decrease in POSTN levels. They hypothesized that POSTN is potential promising marker for assessment of pregnancy outcome [33]. Both presented studies have consistent results, however, their serious limitations are small study groups which should be expanded to strengthen conclusions.

In an interesting paper, Song et al. [34] presented proteomic analysis of neonatal umbilical cord serum. They disclosed differences in protein expression between fetal gender. They concluded that POSTN expression is consistent with the marked rate of growth and development of the fetus. Moreover, they identified 61 neonatal specific proteins that were absent in the adult plasma proteome, *i.a.* POSTN. It seems contrary to the results of many other research teams, who demonstrated presence of POSTN in adults' sera. Song et al. [34] analyzed arterial umbilical blood obtained during vaginal term delivery. Placental ablation may potentially influence the POSTN level so at least venous umbilical blood should be checked. Optionally, blood from cordocentesis would be particularly useful for comparative study. The results should also be analyzed bearing in mind the study by Sasaki et al. [35] in 2002 who speculated POSTN role in the pathogenesis of preeclampsia. They tested blood by novel sandwich chemiluminescence assay as well as placenta by using RT-PCR and *in situ* hybridization of preeclampsia and normotensive pregnant women. They found elevated serum POSTN levels and its increased expression in placental stromal cell. The authors hypothesized that serum POSTN might be a novel marker of preeclampsia in mechanism of release from the placenta due to disturbed adhesive interactions between cells or as an element of inflammatory process connected with activation of leukocytes and endothelial cells. The exact function of POSTN remains unclear, but the fact that it is an adhesion molecule in view of achieved results suggest novel mechanisms in preeclampsia.

Regarding known functions and interactions of POSTN there are some potential clinical implications which should be evaluated. Dobreva et al. [36] decided to investigate bone morphogenetic protein (BMP) target genes, putative amniotic membrane markers in mouse. There is evidence that deficiency in one of several components of the BMP signaling cascade in mice resulted in defective development of the early amnion. In comparative gene expression analysis of acknowledged target genes for BMP in different extraembryonic tissues, they disclosed by *in situ* hybridization POSTN mRNA enrichment in amnion throughout gestation. The Authors suggested usage of POSTN and protein AP-2 as a transcriptional signature for different mouse extraembryonic tissues. Until now, no human studies were conducted. Moreover, Ivancsó et al. [37] performed a study analyzing plasma levels of POSTN in pregnant women with asthma, as there is already strong evidence that POSTN is a useful biomarker in asthmatic patients. The authors stated that POSTN correlates with lung function in asthmatic pregnancy and it may play role in a normal gestation but should be handled with caution in pregnant women due to the possible influence of pregnancy on its plasma levels. Additionally, Świrska et al. [38] prepared very comprehensive review on selected cytokines and hormones of confirmed or possible role in pathogenesis of gestational diabetes mellitus. They cautioned about substantial facts, *i.e.* known relation between POSTN concentration and metabolic diseases as POSTN enhances c-Jun N-terminal kinase-dependent suppression of fatty acids oxidation in the liver and enhanced POSTN expression by higher glucose concentrations. Moreover, they showed many annotations in the literature which point to the possible involvement of POST in gestational diabetes mellitus. However, surprisingly and sadly in available databases there is lack of studies analyzing mentioned above possible relationship.

## CONCLUSIONS

In this review study we presented current knowledge about periostin expression in the field of gynecology and obstetrics. Many achieved results are interesting and further studies are needed to verify some hypotheses. Structure, signaling pathways as well many functions of periostin are well-described. However, as it was clearly shown there is a lot of unknown issues which are waiting to be explored.

***Conflict of interest***

The authors declare that there are no conflicts of interest.

### *Acknowledgments*

The study performed as a part of the project entitled „Expression of periostin in invasive and preinvasive cervical lesions" and financed by grant of the Polish Society of Gynecologists and Obstetricians in Young Researcher Competition.

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VIA MEDICA

RECOMMENDATIONS

Ginekologia Polska
2020, vol. 91, no. 6, 352–361

ISSN 0017–0011

DOI: 10.5603/GP.2020.0081

# Recommendations of the Polish Society of Gynaecologists and Obstetricians for removal of the uterus by vaginal, laparoscopic and abdominal routes

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## ABSTRACT

The recommendations represent the current procedure, which may be modified and changed where justified, after a thorough analysis of the given clinical situation, which may be the basis for their modification and updating in the future.

**Key words:** hysterectomy; transvaginal; laparoscopic

Ginekologia Polska 2020; 91, 6: 352–361

### Objectives

The purpose of these recommendations is to develop indications for the removal of the uterus by vaginal, laparoscopic and abdominal routes. The guidelines are based on the latest literature reports and the experience of the authors.

## INTRODUCTION

A hysterectomy is one of the most frequently performed gynecological procedures and one of the most frequent elective operations in the world. Uterine removal can be performed in several ways: transabdominal (TAH/AH), laparoscopic (LH), vaginal (TVH/VH) and robot access [1–3]. Indications for a hysterectomy for non-cancerous reasons are most often: symptomatic uterine myomas, abnormal bleeding from the reproductive tract, endometriosis or genital depression/exclusion [4, 5]. According to ACOG, transvaginal removal of the uterus is the safest and most cost-effective method of hysterectomy for non-cancerous indications [1]. Laparoscopy also has many advantages, such as image magnification providing better visualization of anatomical structures and identification of the disease outside the uterus. It also allows better access to retroperitoneal spaces thanks to pneumodissection [6, 7]. Both types of minimally invasive surgery (VH; LH) compared to transabdominal surgery is associated with shorter surgery time, less blood loss, fewer transfusions, shorter hospital stays, fewer used painkillers, faster return of intestinal work, shorter recovery time and faster return to normal daily life and work activities [5, 8]. All these factors make these methods of uterine removal much more beneficial also in socio-economic terms [9].

Despite these undisputed advantages, both in Poland and worldwide, the dominant way of performing a hysterectomy is still via laparotomy. This may be due to the lack of appropriate emphasis on minimally invasive operations during the training of gynaecology and obstetrics specialists, the lack of clear guidelines enabling appropriate qualification for this type of procedures, the habit of operators or finally the lack of knowledge of patients about operating techniques [10, 11].

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According to NHF (National Health Fund) data, 35,025 hysterectomies were performed in Poland in 2009, of which as many as 22,526 (64%) were performed via the abdominal tract (TAH), of which 10148 (28%) — supracervical amputations (TASH), and only 1740 (5%) — vaginal tract (TVH), and 611 (1.8%) — laparoscopic (LASH/TLH). In 2013 the percentage distribution of methods of performing hysterectomies practically did not change: abdominal surgery – 16,443 (53%), including TASH — 11,655 (38%), TVH 1292 (4%), LASH/TLH — 1,582 (5%). As we can see, minimally invasive surgeries (TVH, LASH/TLH), despite their advantage over transabdominal surgeries, are still performed in Poland in a significant minority of cases (less than 10% hysterectomy).

## Indications for removal of the uterus for non-oncological reasons

There are a number of indications for uterine excision for non-oncological reasons. The choice of an appropriate surgical method should depend on the type of condition (Tab. 1).

One of the most common indications for hysterectomy are abnormal or not responding to hormonal treatment uterine bleeding. The basis for the diagnosis of the disease is exclusion of uterine cavity and endometrium pathology. Prior to surgical treatment, patients with abnormal uterine bleeding should undergo laboratory diagnosis (evaluation of blood morphology and biochemical parameters), imaging (*e.g.*: ultrasound examination and optional hysteroscopy) and fractional abrasion from the canal and uterine cavity. In case of normal laboratory results and non-oncological histopathological examination, hormone therapy (progestogens, E+P or tibolone) is indicated for at least three months. In the absence of improvement after pharmacological treatment, the patient is a candidate for a hysterectomy, of course depending on age and procreation plans.

The second and most common reason for the patient's qualification for a hysterectomy are myomas. Most women with uterine myomas complain of abnormal, heavy and irregular uterine bleeding (30%) and abdominal pain (39%). In asymptomatic patients, the diagnosis is based on a two-handed gynecological examination and ultrasound diagnostics. Sometimes it is also helpful in the assessment of myomas to extend the diagnosis by magnetic resonance imaging or, less frequently, computed tomography.

Patients with asymptomatic uterine myomas do not require any treatment but only systematic gynaecological control at six-month intervals. Indications for surgery due to uterine myomas are ineffective non-operative treatment of myomas and recurrence of symptoms after non-operative treatment.

When choosing the surgical technique for a hysterectomy, the size and volume of the uterus is important and not the number of myomas. According to the ACOG recommendation of 2009 in patients qualified for a hysterectomy, the size of the uterus not exceeding 12 weeks of pregnancy (about 280–360 g) is an optimal indication for a transvaginal hysterectomy [12]. For larger sizes (volumes) of the uterus, the laparoscopic route (LH) is a better choice. The last option (difficult or risky laparoscopic technique, low operator experience) should be considered for an abdominal hysterectomy.

There is no doubt that as experience is gained in vaginal techniques, the size of the uterus over 12 weeks of pregnancy and the volume over 300 $cm^3$ is not contraindicated for a vaginal hysterectomy. As proved by Cho HY et al. [13], transvaginal method is safe even if the uterine weight exceeds 500 g — the most important in choosing this surgical route is the experience of the operator. In the case of large uteruses, an important factor in the choice of the vaginal route is its vaginal "availability", *i.e.* such factors as uterine size, mobility (possible lowering), vaginal size and the angle between the cervix and the corpus. Vaginal width can be assessed by physical examination — the wider and shorter the vagina, the easier access to the uterus will be and the surgical field will be more visible [11, 13, 14]. The angle between the cervix and the corpus can be assessed during ultrasound (Fig. 1). If the angle between the lateral boundaries of the uterus is greater than 140°, it facilitates the gynaecologist's vaginal excision. An angle of less than 90° makes vaginal access to the uterine vessels and the uterine shaft difficult or even impossible.

The location of the myomas must also be considered when accessing the way of excision of the uterus in case of myomas. In the case of large size myomas (more than 6 cm in diameter) with a subcutaneous location on the anterior wall of the uterus shaft, the vaginal hysterectomy may be, for less experienced operators, very difficult or even impossible to perform due to the low downward retractability of the uterus (blocking the uterus on the pubic bones). It is worth noting that the method of choice in the surgical treatment of submucous fibroids is surgical hysteroscopy [15].

**Table 1. Indications for uterine removal for non-oncological reasons**

| | VH | LH | AH |
|---|---|---|---|
| Functional uterine bleeding | 1 | 2 | |
| Intrauterine adenomyosis/Adenomyosis | 1 | 2 | |
| Uterine fibroids: uterus size < 12 hbd | 1 | | |
| uterus size 14–16 hbd | 2, 1* | 1 | 3 |
| uterus size 17–20 hbd | 1* | 1 | 2 |
| uterus size > 20–24 hbd | 1* | | 1 |
| Recurring endometrial polyps | 1 | 2 | |
| Endometrial hyperplasia (with or without atypia) | 1 | 2 | |

1 — first choice; 2 — second choice; 3 — third choice; * for experienced gynaecologists

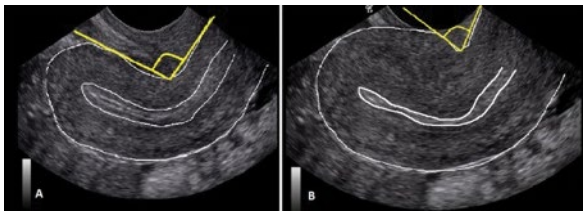

**Figure 1.** Relationship between the size of the uterus and the angle of the lateral boundaries of the corpus uteri and the cervix
A— uterus of correct size; B — enlarged uterus

A particular situation and indication for uterine excision is adenomyosis. Adenomyosis, i.e. endometrial adenomyosis, is characterized by the presence of endometrial tissue and bedding outside the uterine mucosa. Often the only and final basis for diagnosis and differentiation from abnormal uterine bleeding is an in-depth histopathological examination of the excised uterus. The adenomyotically altered uteruses are often enlarged and sometimes heterogeneous. In the case of significantly enlarged uteruses, an experienced gynaecologist can perform a vaginal hysterectomy, using appropriate techniques of morcellation of this organ.

Another indication for a hysterectomy is painful menstruation, which often accompanies adenomyosis or endometriosis of the smaller pelvic organs.

With ineffective analgesic and hormonal treatment, uterine excision seems to be the only effective method.

There is also a group of patients suffering from chronic pelvic pain.

In a significant percentage of cases the pain is associated with severe peritoneal endometriosis (usually with the presence of intraperitoneal adhesions) or with the presence of deeply infiltrating endometriosis. Although in these situations uterine excision seems to be controversial, after using all available pain treatment methods, hysterectomies give hope for improvement of ailments and well-being.

An important indication for uterine excision is the overgrowth of the uterine mucosa (endometrium). The therapeutic management depends on the histopathological outcome of the scrawls from the canal and uterine cavity. Uterine excision seems unjustified in cases of simple proliferation due to lack of evidence of endometrial neoplasia. Patients with complex endometrial proliferation without atypia should initially undergo a three-month hormone therapy containing progestogens, and then have the canal and uterine cavity fractionated abrasion performed again. The initial hormone treatment in this case is justified due to the low risk of disease progression. If a repeated histopathological examination shows endometrial proliferation again, the uterus should be excised. In the case of complex endometrial proliferation with the atypia, treatment of choice is the operation of uterine removal.

Additional attention is required for patients with severe dysplasia or cervical cancer in situ. Routine hysterectomy due to dysplasia or pre-invasive cervical cancer is not recommended. The choice of treatment should consider the severity of the disease, the age of the patient and maternal plans. For those patients who do not want to have more children or those with a relapse or after an unfortunate excision of the lesion, it is best to remove the uterus. Early cervical cancer of grade Ia1 (without occupying vascular and lymphatic space) requires nothing more than a simple hysterectomy. The choice of laparoscopic or abdominal route is not recommended if no pathology is found in the appendages.

Rare indications for uterine excision are also recurrent stem and cervical polyps.

Table 2. shows general and indicative "tactical" assumptions in the choice of the path to uterine excision. They may

**Table 2.** Choice of hysterectomy for non-oncological reasons

| **Choice of hysterectomy method** | |
|---|---|
| Vaginal hysterectomy (VH) 70–80% | |
| Abdominal hysterectomy (AH) 5% | Laparoscopic hysterectomy (LH) 15–25% |
| • lack of experience in vaginal and laparoscopic operations<br>• coexistence of non-gynecological pathology*<br>• large uterus over 20–24 weeks of pregnancy*<br>• pathology of the appendages*<br>• extensive/massive intraperitoneal adhesions — status post numerous abdominal surgeries* | • necessity of abdominal inspection<br>• coexistence of non-gynecological pathology*<br>• large uterus over 16 weeks of pregnancy*<br>• the need to remove appendages*<br>• pathology of the appendages*<br>• advanced endometriosis, deeply infiltrating endometriosis<br>• extensive/massive intraperitoneal adhesions (PID, status post abdominal surgery, abdominal pain)<br>• narrow vagina<br>• nullipara/no vaginal delivery*<br>• immobile uterus<br>• no access to the cervix (status post cervical amputation/very small cervix)*<br>• lack of experience in vaginal operations |

* — commentary in the text of the paper

be a guideline for gynaecologists training in vaginal and laparoscopic hysterectomy techniques. According to the recommendations of most gynaecological scientific societies and our own experience, a vaginal hysterectomy is possible in 70–80% of cases of indications for uterine excision. An abdominal hysterectomy (laparotomy) should be performed only in about 5% of cases of indications for uterine excision for non-oncological reasons. The remaining 15–25% of hysterectomies should be performed laparoscopically. The laparoscopic technique should be used when vaginal access is difficult and the operator's experience is insufficient in the case of nullipara, large uterus (size 16 weeks is usually the upper limit of TVH) when uterine mobilization is insufficient [16].

To meet such guidelines, in addition to adequate training in vaginal and laparoscopic hysterectomies, it is necessary to completely change the mentality during the qualification process for the decision on the method of uterine excision. If the decision has aready been made to remove the uterus, the vaginal route should be considered first. The alternative way to remove the uterus should only result from contraindications or serious difficulties in the vaginal tract.

So, when is the appropriate time for a laparascopy or laparotomy? A few remarks from the commentary to Table 2. The coexistence of non-gynecological pathology may be an indication for laparotomy or laparoscopy. The decisive factor in this case will be the experience of the surgeon who will operate such a pathology.

Myomas is almost a classic indication for a vaginal hysterectomy. For an experienced vaginal surgeon, the size of the uterus even above 1 kg will also not be a contraindication for such surgery. However, in the initial period of a vaginal hysterectomy, a laparoscopic route should be considered for uterus sizes over 16 weeks of pregnancy, and a laparotomy should be considered for uteruses over 20–24 weeks of pregnancy.

During a vaginal hysterectomy most often the fallopian tubes and ovaries can be removed without major problems — this can be done in 70–85% of cases. The decision to remove the ovaries and/or fallopian tubes is independent of the method of hysterectomy and is not contraindicated for transvaginal removal [11, 17]. In the case of absolute necessity to remove adnexa during a hysterectomy (*e.g.* endometrial carcinoma G1), the route or laparoscopic assistance should be considered.

The coexisting pathology of appendages is an indication for laparoscopy or laparotomy. The choice of surgical route depends on many factors, including the nature of the lesion (solid, fluid, etc.), vascularisation, size, laparoscopic experience, etc. If the operator has doubts about the transvaginal hysterectomy due to ovarian or fallopian tube diseases, deep endometriosis or adhesions, it is acceptable to visualize the pelvis smaller with a laparoscopic probe. This will allow the anatomy to be assessed and a final decision to be made on how to operate [10, 12].

A difficult decision to choose the route of uterine excision will depend on a patient after many abdominal surgeries, especially surgical, intestinal (often with a history of postoperative bowel obstruction). In this case extensive intraperitoneal adhesions, intraoperative intestinal damage and postoperative intestinal obstruction can be expected. Both laparoscopy and laparotomy are exposed to such complications. Sometimes in such cases it is worthwhile to choose the vaginal route bearing in mind that the operation takes place largely outside the peritoneum, the adhesions usually cover the bottom of the uterus shank, which means that we do not have to remove the adhesions to the same extent as during laparoscopy or laparotomy (operations from the top, *i.e.* before we get to the uterus we have to remove the adhesions and intestines), so the risk of postoperative obstruction is much lower.

For an experienced vaginal surgeon, a situation of lack of birth by vaginal route (or nullipara) and lack of access to the cervix (*e.g.* very small cervix, condition after cervical amputation) are not contraindications for vaginal excision. In most cases, a vaginal hysterectomy can be performed in women who have not given birth or have undergone C-section [18, 19]. According to Tohic AL et al. [20], even in 92% of women in this group, this route of uterine removal may be successful. It should be noted that the lack of access to the cervix (*e.g.* the very small cervix, the condition after cervical amputation) will also be a major obstacle for laparoscopic hysterectomy, as the insertion of a collar-type uterine manipulator onto the cervix may be difficult or impossible.

## Technique of excision of the uterus through the vaginal tract (TVH)

The patient is placed in a lithotomy position for a transvaginal hysterectomy. Attention should be paid to the preparation staff to ensure good access to the surgical field — the buttocks of the patient should be placed slightly beyond the edge of the operating table, preferably the lower limbs should be bent in the hip joints at least 90 degrees. This is not always possible, especially in elderly patients or patients with orthopaedical diseases of this area of the body. When arranging the patient, excessive bending and abduction of the thighs should be avoided, as this may lead to temporary or permanent nerve damage caused by such positioning — especially pressure on the lateral surfaces of the lower leg should be avoided due to the risk of fibular nerve damage.

Once the surgical field is decontaminated and draped, the patient is obligatorily catheterized; if left during surgery, the catheter is usually placed on the lower surface of the

patient's left thigh. The displacement of the catheter over the groin should be avoided — this often leads to blockage of urinary outflow and lack of control over diuresis. After anaesthesia, an examination of the patient is carried out to check the degree of uterine prolapse, vaginal width and the presence of possible changes in the pelvis minor other than myomas.

After the insertion of bivalve speculum (most often Kallmorgen speculum, less frequently Dever or Haney's retractors) and after the cervix is visible, the vaginal part of the cervix is fastened with bullet forceps. In the case of significant anatomical damage to the cervix (extensive cracks up to the vaults), the bullet forceps should be fastened so that the shape of the cervix remains its anatomical form as much as possible. In patients with prior cervical amputation, the cervical stump should be grasped in such way that the vaginal vauts can be dissected and access to the fastening point of the bullet forceps be gained without the risk of the walls of the bladder being drawn into the bullet forceps and then into the operating field. In these patients, it is recommended to inject the vaginal wall with a saline solution or ready-made solutions of 1% or 2% lidocaine or 0.5% bupivacaine with an adrenaline solution titrated to 1:200,000. In practice, preparation of the solution for hydro-dissection consists of adding to 20 mL of local anesthetic solution (lidocaine, bupivacaine) or 20 mL of saline solution 4 drops (about 200 microliters) of 0.1% adrenaline solution. Please note that the maximum amount of lidocaine should not exceed 7 mg/kg or a total of 500 mg for a healthy adult and the amount of bupivacaine should not exceed 225 mg. Practically during transvaginal hysterectomy the total volume of hydro-dissection solution does not exceed 10 mL. This procedure significantly facilitates the preparation of the space between the bladder and cervical stump. The injection procedure described above should not be used routinely in each patient as it may significantly change (shift) the site of the original vaginal wall incision. In order to determine the position of the bladder, a manoeuvre consisting in slight overhead pushing of the cervix may be performed — the position of the bladder wall is indicated by a fold of the vaginal wall (bladder reflection).

In the case of non-oncological transvaginal hysterectomy, it begins at an incision of the vaginal wall in the immediate vicinity of the cervix (maximum 1.5–2 cm from the external cervical os) with maintaining the peri-cervical ring structures (equivalent to the transabdominal uterine excision by Aldridge's method). In patients with oncological conditions (*e.g.* ca endometrial low grade, cervical dysplasia) the distance of the vaginal wall incision from the external cervical os depends on oncological indications and is the responsibility of the operator. If a significant vaginal fragment has to be excised (*e.g.* extensive severe dysplasia), the risk of bladder damage during the preparation from both the vaginal wall and cervical side should be kept in mind.

The first incision of the vaginal wall at the cervix should be made perpendicular to the axis of the cervix up to the cervical stroma — at the right depth of the incision, the vaginal wall will usually separate from the cervix by itself. The vaginal wall incisions are usually made with monopolar tools, in which we use a coagulation module or, less frequently, with a scalpel. Vaginal walls are usually incised in a circular manner, some operators in patients with a large uterus make an incision of the posterior vaginal wall in which the sharp end of the letter V reaches the posterior vaginal vault (Benenden incision) to enlarge the access field to the uterus. A very helpful tool at this stage of the operation is the Breisky retractors.

After cutting the vaginal walls and gaining access to descending bunches of branches of uterine vessels, usually with power tools closing blood vessels or sutures, cervical vessels (branches of descending uterine vessels) should be ligated. Modern medical technologies offer power tools for closing large (up to 7 mm in diameter) blood vessels (vascular sealing) and have significantly influenced surgical possibilities, even contributing to changes in surgical procedures.

In the next stage of surgery, we gently move the front and back vaginal wall upwards, gaining access to the front and back fold respectively. An important practical tip is to find the right layer of preparation, both in the anterior and posterior compartment — then it is an avascular space and we practically do not observe bleeding. Of course, in the case of a large uterus, the preparation of peri-cervical tissues is multistage, but as a rule we should strive to open the posterior peritoneal fold (pouch of Douglas) first. It is helpful at this stage to find and identify the sacro-uterine ligaments — between them the peritoneum of Douglas's fold can be easily identified and after grasping it with tweezers, cut with scissors. After opening the peritoneal cavity of the posterior fold, we often observe the leakage of a small amount of serous fluid and very clearly tension the sacro-uterine ligaments. After inserting the Breisky retractor into the posterior peritoneal cavity, easily under visual control, we coagulate/split and cut the sacro-uterine ligaments. This manoeuvre gives a very good view and access to the cardinal uterine ligament, which after coagulation/puncture in its lower part we cut off. At this stage of the operation we obtain good uterine mobility that enables the next steps of the operation. The opening of the anterior peritoneal fold (vesico-uterine pouch) is a more difficult stage than the opening of the posterior fold because the parietal peritoneum adheres, sometimes very closely, to the uterus. It is helpful to lift the parietal peritoneum with surgical tweezers and cut under

visual control. Other manoeuvres (*e.g.* blunt-finger/gasket preparation) are most often ineffective and contribute to non-physiological peritoneal detachment, the creation of a large dead space and greater postoperative pain. Bleeding from the posterior vaginal wall cuff is quite common — the bleeding is controlled either by cauterisation or by placing a continuous suture (1–0) on the vaginal edge. After gaining access to the anterior wall of the uterus, we continue coagulation/ pricking and cutting off the lateral perimetrium up to the height of the tray of the round ligament of uterus and proper ovarian ligament. All these structures are coagulated/ pricked and cut off in turn. Operators using the traditional technique with vessel pricking stitch the peduncle of the cardinal uterus ligament to the peduncle of the sacro-uterine ligament. In the power tool technique, both these stumps are separate structures.

If the patient qualifies to cut out the fallopian tubes, this can be done at this stage of the operation by finding the abdominal os of the fallopian tube, grasping this part of the fallopian tube with the window forceps and by successively coagulating the mesentery of the fallopian tube and cutting off as close to the fallopian tube as possible, mobilising this organ up to its intramural. This allows removal of the fallopian tube in one tissue block with uterus. In the case of a tight operating field (large uterus) this can be done after the removal of the uterus from the vagina — then it is worthwhile to seal the stump of the appendages with ticks or sutures in order to find them later in the operating field and pull them downwards. In this way it is also possible to remove the pathology of the ovary and, depending on the indications, enucleate the cyst out or remove the ovary(s).

In the case of very large uteruses, a slightly different preparation may be used, involving the maximum release of the cervix (under the given conditions) and then cutting off the cervix. This gives access to the uterus corpus, which becomes much more mobile under these conditions. We continue the preparation of the lateral perimetrium, on the principle of turning like a ball, of the uterine corpus, sometimes changing sides several times (right and left). It is recommended to use power tools to close the vessels instead of the traditional technique of grasping and pricking individual anatomical structures with ticks, as this significantly reduces postoperative pain (less use of analgesics) and reduces tissue reaction, causing faster tissue healing.

In the case of large uteruses, it is necessary to reduce the size of the surgical preparation. Several morcellation techniques can be used. The most common technique is the so-called "coring" technique, which consists in conical incision of the corpus until the size of the uterus can be removed by the vagina. This method is most recommended for safety reasons (protection of the bladder and rectum) and lack of contact of myometrium/endometrium with the peritoneal cavity. In the case of large anatomical deformities of the uterus, usually because of myomas, morcellation is also used but dictated by anatomical conditions. It is worth thinking, in the case of myomas, about intraoperative exfoliation of myomas as a method to reduce the size of the uterus. It also happens that the myoma(s) are left unintentionally in the peritoneal cavity — this phenomenon is observed in the case of myomas which are scattered, detached during pulling the uterus down and "dying" from the operator's field of view in the intestinal loops.

After obtaining and checking the haemostasis, we begin to close the peritoneum and vaginal stump. For this purpose, Kocher's forceps should be used to grasp the stumps of the sacro-uterine ligaments and the front vaginal wall in the midline. While the peritoneum of the back vaginal wall is usually well visible and easy to grasp, peritoneum of the vesico-uterine pouch needs to be found — sometimes its edge is quite deep in the pelvis minor. The vaginal stump is usually stitched transversely by means of a continuous stitch, ensuring that the anterior and posterior peritoneal wall suture line is gripped — this reduces the volume of the so-called dead space, thus reducing the risk of postoperative adhesions or hematoma.

In some centres, the suture of vaginal walls after surgery is used longitudinally, in the sagittal dimension — it is not supposed to change the length of this organ, although the literature data do not prove the rightness of such an approach.

In patients with prior lowering of the uterus and posterior vaginal wall it is worth using McCall's external sutures, which significantly restore proper anatomical conditions and reduce the risk of vaginal stump prolapse in the future.

In patients with no pelvic floor static disorder, no prophylactic surgery is recommended, based on the assumption that correctly grasped and sewn together with the anterior vaginal wall, the sacro-uterine ligaments and the paracervical ring preserved during the vaginal hysterectomy is sufficient to protect the pelvic floor static after this procedure. After cleaning and disinfecting the surgical field, it is recommended to place the seton in the vagina and maintain it together with the Foley catheter until the next day. The patient is usually discharged home on the first, less frequently on the second postoperative day. Postoperative control is recommended 4–6 weeks after the surgery — if the surgical suture is still present, we should gently, usually with tweezers, remove fragments of this material as it no longer has any mechanical functions but is only an irritating element and may contribute to the production of bleeding postoperative granulation tissue. The next control is recommended about 6 months after the operation and is intended, in addition to a routine gynaecological examination, for urogynecological evaluation (possible disorders of miction or pelvic floor static disorders).

Additional notes:

1. Assessment of pelvic floor static disorders and possible prevention of POP.

During transvaginal hysterectomies, the posterior vaginal wall statics should be routinely assessed, because in the final stage of transvaginal excision surgery the defects of the posterior, central and anterior compartments can be relatively easily and effectively provided. The most used method is McCall's culdoplasty procedure. In case of significant prolapse of the posterior vaginal wall (POPQ > 2) it is recommended to put 2–3 McCall's internal and external sutures, according to the technique described by this operator. The number of McCall's external sutures depends on the degree of prolapse and anatomical conditions of the operated person (usually 1 or 2 sutures). McCall's external sutures are tied only after the vaginal walls are sewn. This surgical technique carries a slight risk of ureter displacement and disturbance of their function (kinking) — therefore, caution should be exercised both in the number of sutures and their location, especially in the cephalad section. Some authors recommend intraoperative cystoscopic control of ureter function (urine jet) — however, this is not an obligatory procedure, especially when fixing small defects in the posterior compartment. In the case of lowering of the anterior vaginal wall, before stitching the cuff, it is also possible to perform a native plastic surgery of the anterior vaginal wall (various surgical techniques). Synthetic materials (mesh) are not recommended due to the high risk of erosion of the prosthetic material.

2. In the light of the available literature data, it is not recommended to routinely use any prophylactic procedures for POPs — patients without any preoperative and during the procedure of pelvic floor static disorders do not require prophylactic POP procedures.
3. Difficulties in opening the anterior and posterior recesses.

Sometimes opening the rear and front recess can be a real challenge for the operator. Most often it is related to past Caesarean section(s) (anterior recess) or past pelvic inflammation of the minor or deep infiltrating endometriosis (DIE) of the rectovaginal septum (posterior recess). In these clinical situations, the preparation in both compartments should be performed acutely, avoiding blunt tissue delamination and following the principle of preparation as close to the uterus as possible. This method of preparation significantly reduces the risk of cystotomy or colorectal damage.

4. Intraoperative damage to the smaller pelvic organs (intestines, bladder).

If an unintentional cystotomy occurs, it should be used, through palpation, to determine the optimal entrance to the anterior cavity. The hysterectomy should then be completed and the cystotomy should be closed, after prior control (transvaginal or cystoscope) of the location of the ureter and bladder triangle discharges. Cystotomy is performed according to the rules of vesicovaginal fistulas surgery by inserting two layers of absorbable sutures with suture 3–0. The catheter in the bladder is removed on day 7–10 after surgery.

If the large intestine (rectum, sigmoid) is damaged, it should also be equipped with two layers of single sutures (3–0) running parallel to the axis of the intestine — this does not lead to postoperative stenosis at the site of postoperative scarring. If the bowel injury is less than 50% of the circumference, no stoma decompression is required.

Occasionally, extremely rare small bowel lesions can also be operated transvaginally after a previous, symmetrical to the site of the lesion, mobilization of at least 10–15 cm of the bowel loop.

## Technique of the laparoscopic excision of the uterus (LH)

The key to success in the performance of laparoscopic hysterectomies is to perform standard surgical steps in succession.

If the surgeon follows the rules for laparoscopic surgery, the risk of complications is extremely low. The placement of the patient in the correct position and on the appropriate anti-slip mats, the use of the correct instrumentation and the positioning of the trocar are the most important factors. The standard set for TLH should include at least four trocars, two graspers, a laparoscopic dissector, scissors, a medical suction device, a monopolar L-hook electrode, a uterine manipulator, a needle holder and a bipolar instrument. Bipolar instruments with deep vessel coagulation are preferred because they provide a permanent vessel closure of up to 7 mm diameter. After the correct positioning of the patient on the operating table (as in the vaginal technique), decontamination and dragging of the operating field, and the patient's catherization, the next step is to insert the uterine manipulator. Most of the manipulators available are well accepted, easy to use, reusable and durable. The uterus can be moved in all directions, while the tip of the manipulator expands and stretches the vaginal vault, especially when cutting off the uterus from the vagina with a monopolar L-hook electrode. The vast majority of the manipulators are equipped with a ceramic cap, which creates a flat surface that adheres to the vagina and makes it easier to dissect the bladder even after Caesarean section.

After the uterus is clamped with the manipulator, pneumoperitoneum should be produced. Veress needle technique: to insert the Veress needle, the operating table must be in a horizontal position. The Trendelenburg tilt is carried out after the formation of the peritoneal emphysema and the placement of the optical trocar. The most common entry point for the Veress needle is the umbilical cord plate, because the layers of the abdominal wall are thinnest at this

level. Before insertion of the needle, determine the course of the aorta via palpation and identify the bifurcation of the hip vessels [21].

The Veress needle must be checked for valve elasticity and gas flow between 6 and 8 mmHg before use. The needle insertion should be at a 45° angle towards the uterus, with the least risk of damaging large vessels. The abdominal wall should be raised before insertion. In obese patients the insertion angle is close to 90°, while in slim patients the angle is close to 45°. If the first attempt fails, a second attempt should be made before choosing an alternative entry route. Optimally, a pressure of approximately 16 mm Hg should be achieved before starting to insert the optical trocar.

No entry technique is completely free from the risk of gas blockage or damage to vessels, intestines or urinary tract. The Palmer point is the safest laparoscopic entry point, as adhesions are the least likely to occur there. For all patients with a much higher risk of adhesions, after abdominal surgery, including Caesarean section, large uterus, umbilical hernias, large ovarian cysts, or a failed bellybutton entry, Palmer described in 1974 an entry point into the abdomen in the medial collarbone line, about 3 cm below the rib margin. If adhesions are suspected in the left subcostal region, the Lee Huang point should be used, which is located in the midline above the navel and is an alternative to a safe entrance to the peritoneal cavity (Fig. 2) [22].

The next stage is trocar positioning: in the case of a large uterus or high uterine mobility, the optical trocar should be placed above the navel so that the visualization of the operating field is sufficient. For this purpose, the patient should be re-examined after anaesthesia and then the position of the optical trocar should be determined. Auxiliary trocar should be placed after the manipulator has been put on so that the lower trocar is at the height of the uterine bottom after its removal with the manipulator cranially and the additional trocar should be placed either in the middle line above the joint or laterally at a distance of about 7 cm from the lower trocar on the operator's side.

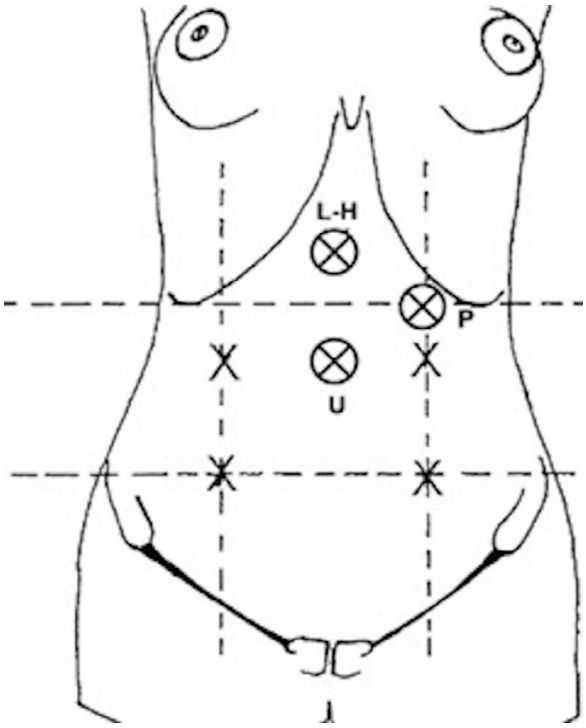


**Figure 2.** Peritoneal entry points and trocar positioning

After the placement of the vision track and working trocars and the insertion of the uterine manipulator, the entire abdominal cavity, uterine mobility, possible pathologies in the area of appendages, adhesions which, despite appearances, even in the epigastrium may impede the operation from running smoothly, should be assessed before the proper procedure. Only after obtaining the optimal working position and proper pressure — usually about 8 mm Hg — can the proper operation begin.

We move the uterus cranially and to the first assistant's side, the assistant grabs the suspensory ligament on the left side by the grasper, the operator cuts the peritoneum showing the retroperitoneal space, and the left ureter. Preparing along the ureter, the uterine vessels are visible. This is helped by positive gas pressure, which penetrates the loose connective tissue and shows the avascular spaces. At this stage it is already possible, although not necessary, for the use of electrocoagulation on the uterine artery at the site of bifurcation.

After separation of the ovarian vessels and incision of the peritoneum of the posterior broad ligament of uterus plate in order to isolate the ureter from possible thermal effects resulting from electrocoagulation, we coagulate the ovarian vessels and cut, then, after coagulation and cutting of the round ligament, the bladder can be separated from the vagina in a safe way, dissecting vesico-uteirne pounch. The uterine manipulator helps us to do this properly tensioned and directed head and towards the bladder. We try to slide the bladder about 1–2 cm so that there is space to put stitches on the vagina. Please note that the vagina is stretched by the manipulator. After it is removed, the vagina shrinks and the margin necessary for proper suture insertion will be correct only when the bladder is dissected.

After lifting the uterus upwards and cranially and to the right side you can safely remove the peritoneum of the back plate of the broad uterine ligament. This manoeuvre causes the ureter to move away and to show the posterior edge of the vagina and the uterine cut-off.

At this stage the uterine vessels on the left side are already visible, which are coagulated and cut above the edge of the collar of the uterine manipulator, so that if additional coagulation is needed a safe distance from the ureter is maintained.

We proceed in the same way on the opposite side, moving the uterus further upwards, this time to the left side.

We cut the uterus from the vagina at the height of the upper edge of the manipulator, starting from the right side at the height of the cut right uterine vessels. Then we go backwards in circles and finally move to the front vaginal wall. This technique allows you to easily identify the vaginal edge. By cutting the back of the vaginal wall first, we do not cause the uterus to fall, and thus it is easier for us to see the uncut front vaginal edge of the vagina and safely cut the uterus from the vagina in the next stage. We remove the uterus and the manipulator through the vagina, then seal the vagina to allow the vagina to suture.

There are many techniques for closing the vaginal stump. Many people use Z-type anchor stitches for vaginal angles and then either continue with single or continuous stitches. The authors prefer a 2–0 continuous self-anchored seam. It is important to always check that the vaginal mucous membrane has been sewn during closing. Difficulties in the continuous stitching are most often found in the opposite corner. If the vagina is not well prepared then you should think about putting on a single seam, it will be easier and safer.

After the removal of the uterus and suturing of the vagina, we always carry out an inspection of the abdominal cavity with particular emphasis on the ovary and uterine vessels. First, we remove the working trocars, evacuate the excess gas and remove the optical trocars under a laparoscopic control.

Additional notes:

Intraoperative damage to the smaller pelvic organs (intestines, bladder).

The most common complication of a laparoscopic hysterectomy is damage to the urinary system. Bladder damage occurs most often during the preparation of the anterior wall and cervix, especially in patients after previous Caesarean section. The cystotomy is delivered laparoscopically with two layers of soluble sutures 3–0, then we check the tightness of the suture using a cystoscope. The Foley's catheter is left in the urinary bladder to a minimum of 7 days after the procedure. A very important element of laparoscopic uterine removal is to visualize the course of the ureter, which reduces the risk of its damage. Depending on the operator's experience, the ureter can be supplied laparoscopically with a cystoscopy and a probe inserted into the ureter. Whenever after LH we are not sure of the continuity of the urinary tract, we should use a cystoscope. It is not necessary to do this routinely after each laparoscopic hysterectomy [23, 24].

Bowel damage is a less common complication. Depending on the location of the damage, its extent and the experience of the operator, it can be delivered by laparoscopy or laparotomy [25].

## SUMMARY

Hysterectomies are one of the most common gynaecological operations. We know different ways to perform them, including minimally invasive methods, which are not only safer but also economically more beneficial than laparotomy. An appropriate look at the selection of the surgical method gives the possibility to reduce the number of hysterectomies for non-oncological reasons from transabdominal access. Training of personnel in transvaginal and laparoscopic techniques should be extensive among obstetrics and gynaecology adepts so that the selection of the least invasive method of uterine removal is not limited by lack of skills.

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RECOMMENDATIONS

Ginekologia Polska
2020, vol. 91, no. 6, 362–371

ISSN 0017–0011

DOI: 10.5603/GP.2020.0075

# COLPOSCOPY 2020 — COLPOSCOPY PROTOCOLS
# A Summary of the Clinical Experts Consensus Guidelines of the Polish Society of Colposcopy and Cervical Pathophysiology and the Polish Society of Gynecologists and Obstetricians

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**The Consensus was developed by clinical experts**
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**The Working Group No. 1 of the Colposcopy Protocols**

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## ABSTRACT

The Polish Society of Colposcopy and Cervical Pathophysiology and the Polish Society of Gynecologists and Obstetricians provide comprehensive guidelines for colposcopy practice in secondary cervical cancer prevention in Poland. This part of the guidelines, developed by the clinical experts of the Working Group No. 1 (WG1), concerns the colposcopy protocols with the main aim of algorithmizing the procedure, together with all procedure-related processes. The detailed analysis of strong scientific evidence and an extensive literature review of current international colposcopic recommendations were carried out, with also a broad investigation of recently ongoing dynamic changes in national health systems. The attention to colposcopic limitations also occurring in Polish conditions was kept. The overriding goal was the recommended obligatory minimal colposcopy approach introduction. To enhance the standard of colposcopy, adjustment of a precolposcopic assessment, a performance technique, types of used biopsies, as well as the procedure documentation was made. Elements of the risk-based stratification for the increased risk of developing cervical cancer was also included if it was applicable for that part of the guidelines. Comprehensive colposcopy guidelines are a step towards the ongoing era of a precision medicine in cervical cancer prevention in Poland.

**Key words:** colposcopy; cervical biopsy; cervical cancer prevention; colposcopic practice; guidelines

Ginekologia Polska 2020; 91, 6: 362–371

*The recommendations present current management that can be modified and changed in justified cases, after careful analysis of a given clinical situation, which in the future may constitute grounds for their modification and updating.*

## INTRODUCTION TO COMPREHENSIVE GUIDELINES FOR STANDARDS IN COLPOSCOPY "COLPOSCOPY 2020"

Colposcopic examination is one of diagnostic-therapeutic key points in the cervical cancer screening (CCS) [1, 2], regardless of the primary screening test used. Histopathological "gold standard" detection of high-grade squamous intraepithelial lesions and cervical cancer is based on colposcopy [2].

The main purpose of these comprehensive colposcopy guidelines is the algorithmization of all processes accompanying this procedure, in achieving the highest possible sensitivity and specificity in Polish conditions [2–4]. In the recommended adjustment of the indications, implementation and colposcopy technique, a variety of currently used approaches in Poland, as well as varying levels of training and experience of colposcopists was considered.

The limitations of a diagnostic value of colposcopies are widely known [2, 3], unfortunately they are far from expected. The sensitivity of colposcopies for detecting high-grade cervical squamous intraepithelial lesions, with subcategorization to cervical intraepithelial neoplasia grade 3 and greater [HSIL (CIN3+)], ranges from 50 to 65%, depending on the study [5–9].

The Consensus was based on a strong evidence with extensive review of current international colposcopic standards [1–5, 10–38] and on the Committee's own experience. Significant limitations resulting from the insufficient availability of properly standardized research, especially in a Polish population, was also maintained.

Participation in the Consensus of pathologists and gynecological cytopathologists aimed at interdisciplinary analysis of all colposcopy-related processes and a diagnostic background, which is an important factor in the continuous pursuit of precision medicine.

The complexity of the colposcopy standardization in Poland is the coexistence of three CCS models: two financed from public funds, *i.e.* population-based (currently not continued) and opportunistic, and one outside the public system based also on the opportunistic model [39].

The specificities of Polish gynecological prophylaxis, including CCS, is an extraordinarily strong sector of a private medical service, rather unprecedented in other countries, paid by patients' own resources without involvement of insurance companies. Many patients directly paying for health services expect to maximize their health interests, not population-based optimization or cost-effectiveness.

Secondary CCS in Poland in the opportunistic model financed from private funds is not sufficiently standardized and practically takes place beyond effective quality assessment and quality control. The problem is compounded by the lack of comprehensive recommendations for CCS in our country for nearly 10 years [40], and its objective assessment due to the lack of comprehensive statistical data and screening results remaining outside of synthetic records [41–43].

The overriding goal of the Guidelines is to change the current state and introduce an original screening model that will allow to combine a standardized controlled opportunistic private CCS model with a population-based organized CCS model financed from public funds. It seems, in Polish conditions only a mixed screening model gives a chance to achieve the expected minimum 70% screening coverage of women [44], which was indirectly but clearly confirmed by the analysis of the Polish organized population model completed in 2017 [45]. Achieving at least minimal screening coverage will bring Poland closer to the fundamental objective of secondary CCS — reducing morbidity and mortality. The implementation of the above in association with primary prevention of cancer, opens the possibility of its epidemiological elimination [46].

Developing Polish colposcopic guidelines with the minimal recommended colposcopy approach is a necessary step to achieve the objectives.

## General aims of the Guidelines

The most important aims of Comprehensive Guidelines for Colposcopy Standards have been developed, based on the detailed analysis of strong scientific evidences, international guidelines of the highest-authority gynecological societies [2, 16, 37, 38] and on the own experience of the Committee members:

1. These guidelines address the colposcopic examination and cervical biopsy in secondary cervical cancer prevention.
2. They were specifically developed for the Polish conditions, with considering the characteristic features of current CCS models in Poland.
3. Guidelines have been developed as understandable and easy to unambiguous interpretation as possible way, with the attention to uncomplicated popularization and application for educational purposes.
4. Recommended colposcopy approaches enable their effective implementation to national conditions.
5. The main goal was to indicate the minimal practice colposcopy guidelines, with "a nothing below" principle.
6. The optimal and the optional practice colposcopy guidelines were also introduced.

## Basics of colposcopy in the interdisciplinary approach

Strategic for understanding the basics of colposcopic examination is to define the transformation zone (TZ) and the squamo-columnar junction (SCJ). To minimizing limitations of colposcopy, the Committee points the need for extended definition of both terms.

SCJ and TZ are basic dynamic landmarks of the transformation process. Transformation zone is the site for the occurrence of over 90% of cervical precancers, according with the LAST 2012 Project and WHO/IARC 2014 named high-grade squamous intraepithelial lesions (HSIL), and of the cervical cancer [2, 3, 47, 48].

The SCJ is defined as the interface between the stratified squamous and the cylindrical epithelium, and its location in the cervix varies. SCJ is the result of a continuous remodeling process associated with uterine growth, cervical size changes, obstetric history, hormonal status, cervical treatment [49–51], and with a vaginal microbiome as well [52].

The process of a migration of the primary SCJ from the initial endocervical to ectocervical position, often distant from the ostium of the external cervical canal, is a physiological phenomenon of reproductive period.

A gradual replacement of cylindrical epithelium by the stratified squamous epithelium is determined by the metaplasia process, which is initiated in response to the acidic vaginal environment [47, 49, 50].

Metaplasia is an adaptive process usually occurring under the prolonged irritation or hormonal factors. It is replacing one type of mature cell with another [53, 54]. A characteristic feature of cervical metaplasia is its multifocality and the ability to merge smaller areas into larger ones, which has a direct impact on the potential multifocality of precancerous lesions, what might be particularly challenging for colposcopists.

The process of cervical metaplasia begins with the reserve cells lying under cylindrical epithelium. Reserve cells proliferation passing through the phase of immature to mature metaplasia causes creates a new epithelial junction (new SCJ) with cylindrical epithelium. The area between the primary and new SCJ is called the transformation zone [47, 49, 50]. A special feature of reserve cells is their increased susceptibility to HPV infection, which is the fundamental factor in cancer transformation [3].

For the reasons above, documenting the visualization of a new SCJ is one of the most important quality indicators

in colposcopy, as well as a location of colposcopic findings in relation to TZ.

The understanding of TZ and the new SCJ is being aware about their possible multifocal appearance. Transformation zone in histopathological meaning is the area in which squamous metaplasia may appear. Reserve cells initialing that process may apply not only to glandular crypts, whose depth may reach up to 10 mm, but may reach up to the isthmus. Precancerous lesions located in these places might be undetected during colposcopy [48, 55–58].

In the opinion of the Committee's Experts, awareness of the limitations of colposcopy, should result in the use of procedures reducing the diagnostic failure. This applies to endocervical sampling and to the standardization of random biopsy in cases of increased risk of HSIL (CIN2+) [59–61].

Committee members emphasize the possibility of developing two different histologic subtypes HSIL within TZ: classic HSIL, when it develops within mature metaplasia through the intermediate stage of LSIL; and thin HSIL. The latter develops within early metaplasia without the intermediate stage of LSIL, near the new SCJ. Thin HSIL has multifocal character and may coexist with classic HSIL, what all together may hinder a colposcopic examination [57, 62, 63].

## Objectives of Working Group No. 1 on Colposcopic Protocols

WG1 recommends presented colposcopy protocols as a necessary component of diagnostic colposcopy approach. These might be complementary to other current nationwide guidelines or can be their integrated part.

The protocols are aimed at multi-level algorithmisation of the procedure in Polish conditions, focusing on indicating the minimal colposcopy approach.

Guidelines do not include diagnostic-therapeutic excisional procedures: electrical loop (LLETZ/LEEP) and surgical cold-knife. A multi-parameter risk stratification (based on additive analysis of precolposcopic screening tests results with colposcopic image) is recommended to treatment using excisional procedure without preceding biopsy [5].

## Assessment of the strength of the recommendation

In assessing the level of evidence and strength of these guidelines, WG1 adopted the classification used in the "European recommendations for quality assessment in cc screening" [44] (Tab. 1 and 2). Due to the lack of relevant published research on the Polish population, level VI (expert opinions) of strength A (procedure strongly recommended), B (procedure recommended) or C (procedure to be considered but of uncertain importance) was adopted for the all "Colposcopy 2020" guidelines.

**Table 1.** Criteria used to assess the level of reliability of scientific evidence

| Level of evidence: | A criterion description |
|---|---|
| Level I | Consistent multiple randomized controlled trials (RCTs) of adequate sample size, or systematic reviews (SRs) of RCTs, taking into account heterogeneity |
| Level II | One RCT of adequate sample size, or one or more RCTs with small sample size |
| Level III | Prospective cohort studies or SRs of cohort studies; for diagnostic accuracy questions, cross-sectional studies with verification by a reference standard |
| Level IV | Retrospective case-control studies or SRs of case-control studies, trend analyses |
| Level V | Case series; before/after studies without control group, cross- sectional surveys |
| Level VI | Expert opinion |

**Table 2.** Criteria used to assess the strength of the recommendations

| Strength of the respective recommendation: | A criterion description |
|---|---|
| A. | Intervention strongly recommended for all patients or targeted individuals |
| B. | Intervention recommended |
| C. | Intervention to be considered but with uncertainty about its impact |
| D. | Intervention not recommended |
| E. | Intervention strongly not recommended |

## Major and minor screening abnormalities

WG1 recommends the major screening abnormalities terminology in assessment of the pre-colposcopic stage, defined as:

- screening test results implicating immediate colposcopy,

  and following colposcopic lesions:
- minor colposcopic findings
- major colposcopic findings
- findings suspicious for invasion
- nonspecific findings (optional)

Which require the use of extended colposcopic protocol, specified in the guidelines as the optimal protocol.

At the precolposcopic stage, minor colposcopic abnormalities include screening test results, which allow a conservative management in specific conditions, usually with follow-up after 12 months. At the colposcopic stage, minor colposcopic abnormalities include colposcopic findings sufficient for the use of the basic protocol.

More detailed definition of major and minor screening abnormalities of the precolposcopic stage remains outside the WG1 guidelines.

### Excisional and ablative procedures

It was decided that protocols for excisional procedures — LLETZ/LEEP, "cold-knife" and ablative procedures (cryo- and laser ablation) should be developed after a completion of other WGs works, in particular the group working on indications for colposcopy, defining major and minor screening abnormalities.

### Types of biopsy in HSIL (CIN2+) risk stratification

Colposcopy with targeted biopsy remains a diagnostic standard in HSIL (CIN2+) detection and the procedure of choice for making therapeutic decisions. Histopathological examination is the "gold standard" [3].

Indications, the number of taken biopsies and the technique of **targeted biopsy** differ significantly not only on recommendations [2, 16, 37, 38], but also between colposcopists [64].

WG1 recommends targeted biopsy when lesions diagnosed as follows are present:

- abnormal colposcopic findings,
- findings suspicious for invasion,
- suspicious metaplasia,
- other suspicious findings.

Whilst taking more, than one biopsy if needed [1, 3].

Many studies prove the limited efficacy of targeted biopsy, *e.g.* the sensitivity for HSIL (CIN3+) varies from 50 to 65%, depending on the study [5–9]. Targeted biopsy cannot be diagnostically effective enough, especially when precolposcopic major screening abnormalities were diagnosed and no colposcopic abnormalities are found.

**Random biopsy** is accepted as an optimal procedure to increase the sensitivity of colposcopy for detecting HSIL (CIN2+), in cases when no colposcopic abnormalities were found.

Random biopsy is defined as a biopsy from each normal quadrant as 2, 4, 8 and 10 clock position at the new SCJ. If new SCJ is not visible a random biopsy is not recommended.

Random biopsy efficacy for HSIL (CIN2+) varies significantly among different studies, with values ranging from 3.8% to 37.4% [65, 66]. These discrepancies are the result of different definitions of abnormal colposcopic findings — a more liberal the definition of abnormality is used the less diagnostic random biopsy is [60].

WG1 recommends a colposcopic nomenclature in accordance with the 2011 IFCPC, translated into Polish with the IFCPC approval (in press).

Comparison of targeted and random biopsy with p16 immunohistochemical staining in cases of HSIL (CIN2 +), shows that lesions detected in random biopsy: 1) are more often limited to one cervical quadrant; 2) they are less often associated with cytological diagnoses of AGC, ASC-H, HSIL and cervical cancer; 3) are more frequent in women over 50 years; 4) are less frequently associated with HPV 16 infection [67].

Independently, taking more biopsies increases colposcopic diagnostic value, regardless of a colposcopists experience or the patient's clinical status [59, 66].

For targeted and random biopsies, WG1 recommends microbiopsy tool with a cutting width of up to 2 mm, minimizing tissue traumatization and patient discomfort or pain. Taking more biopsies using microbiopsy instrument does not reduce the patient's acceptance of the procedure [61].

Colposcopic sensitivity for HSIL (CIN2) might be substantially increased, in specific clinical cases, by endocervical sampling with a detection rate is up to 16.7% (average 5.5%) [59, 68].

**Endocervical sampling** can be taken by a traditional sharp curette or with endo-Cervex root by vigorous brushing, or by using both methods. Diagnostic value of both method — ECC and ECB — is comparable [59, 68, 69]. Endocervical brushing in most cases does not require dilatation of the cervical canal, so it is a sparing procedure of choice. Endocervical sampling is not recommended during pregnancy [59].

Indications for ECC/ECB including HSIL (CIN2+) risk stratification were listed in the basic protocol.

**Endometrial sampling** (minimally with aspiration biopsy, *e.g.* using pipella device), in combination with colposcopy with ECC/ECB, is recommended in women of 35 years and older with AGC (all subcategories) or AIS in cytology. As well as in younger women with endometrial cancer risk (*e.g.* atypical hyperplasia/endometrial intraepithelial neoplasia in histology, abnormal uterine bleeding [59, 62], symptoms suggesting chronic lack of ovulation [70]).

Recommended by WG1 a general approach for performing colposcopic examination covers:

- a colposcopic assessment of the cervix divided into quadrants with a clockwise manner (quadrant I — front left, II — rear left, III — rear right and IV — front right) to optimize the procedure.
- biopsy from all recommended areas (more than one biopsy if needed), with a rule of thumb — the worst lesion is usually located closest to new SCJ.
- in cases of precolposcopic major screening abnormalities when no colposcopic abnormality was found a random biopsy from each quadrant as 2, 4, 8 and 10 o'clock at new SCJ should be taken.
- endocervical sampling in all non-pregnant patients.

In HSIL (CIN2+) risk stratification, identification at least two major screening abnormalities of cytologic HSIL, positive HPV 16 and/or 18 infection and major colposcopic findings is associated with higher risk of precancers than the occurrence only one major screening abnormality.

Similarly, concurrent cytologic diagnosis less than HSIL, no HPV 16 and/or 18 infection, and no abnormal colpo-

scopic findings is associated with a lower risk of precancer than the occurrence only one minor screening abnormality. Multi-parameter risk stratification increases a diagnostic value of secondary CCS, including analysis the results of colposcopic examination [5].

In the opinion of WG1, new imaging colposcopy technologies require clinical validation before they are introduced into routine colposcopic practice [64, 71, 72].

## Component procedures of colposcopy

According to these guidelines, a full colposcopy procedure should consist of the following components:

1. Precolposcopic assessment.
2. Colposcopic examination with one of recommended colposcopy protocols.
3. Documentation of colposcopic findings.

## Precolposcopic assessment

WG1 recommends precolposcopic assessment with one of two recommended options:

- basic — obligatory minimum for precolposcopic assessment.
- optimal — recommended precolposcopic assessment, optimal at the time of developing draft guidelines.

Assessment parameters for each option are listed in Table 3.

**Table 3.** Options of precolposcopy assessment with recommended parameters

| PRECOLPOSCOPY EVALUATION OPTIONS | BASIC | OPTIMAL |
|---|---|---|
| PARAMETER | – | – |
| Indications for colposcopy | x | x |
| **Status/result of the last HPV/cytology/p16/Ki67 test** | x | x |
| **Status/result of the previous HPV/cytology/p16/Ki67 test** | | x |
| Result of the previous colposcopy | | x |
| Excision/ablative procedures | | x |
| LMP — date or age | x | x |
| **Pregnancy status** | x | x |
| **Obstetrical history** | | x |
| HCT — type | | x |
| IUD — nonhormonal/hormonal | | x |
| Hormonal therapy — type | | x |
| **Menopausal status/age of LMP** | x | x |
| MHT — type | | x |
| **Status post hysterectomy** | x | x |
| Smoking | | x |
| HIV status | | x |
| HPV vaccination — name, number of doses | | x |
| Others — what? | | x |
| **Informed consent of the patient** | x | x |

HPV — human papillomavirus; p16/Ki67 — immunocytochemical test p16/Ki67; HCT — hormonal contraceptive therapy; MHT — menopause hormone therapy; HIV — human immunodeficiency virus; IUD — intrauterine device; LMP — last menstrual period
Basic pre-colposcopic evaluation in **bold**

## Colposcopy examination

As the routine basic colposcopy technique, the following steps are recommended (in the order specified):

1. gross examination of vulva and vagina,
2. initial assessment of the cervix and upper vagina at different power magnifications*,
3. careful (without causing bleeding) application of saline with washing away the mucus,
4. re-evaluation of the cervix and upper vagina at magnification* and with green filter (necessary before applying acetic acid),
5. application of 3–5% acetic acid,
6. examine the cervix and upper vagina at different power colposcope magnifications* [73]:
   a) after 1 minute routinely
   b) after 3 minutes (optionally)
7. selection of lesions for biopsy,
8. colposcopic biopsy,
9. achieving and ensuring haemostasis.

*4 to 15 times magnified image recommended.

Photographic documentation at least of 3), 4) and 6 a) examination steps is recommended, if possible. WG1 points also that photo-documentation a post-biopsy step, can be a useful educational tool.

Due to the inclusion by the IFCPC the Lugol staining result (Schiller test) to non-specific colposcopic images, WG1 does not recommend Schiller test in the routine practice [74].

## Colposcopy protocols — a systemic approach

WG1 recommends one of three levels of colposcopy protocols to use in routine colposcopy practice:

- BASIC — minimal colposcopy approach (obligatory).
- OPTIMAL — recommended colposcopy approach, optimal at the time of developing draft guidelines.
- OPTIONAL — approach accepted by Experts as having the highest diagnostic sensitivity in detecting histologic HSIL (CIN2+) at the time of developing draft guidelines.

The choice of the colposcopy protocol in screening models being founded by public resources is an autonomous decision of the founder.

The key principle for the physician participating in secondary CCS in the era of evidence-based precision medicine is a fundamental care for the patient's health interest based on available experts' guidelines and with the individualization of a management.

**Table 4. Recommended levels of colposcopy approach, including minimal obligatory level for colposcopy practice. Detailed description of each approach in the main body of the guidelines**

- BASIC — minimal colposcopy approach (obligatory)
- OPTIMAL — recommended colposcopy approach, optimal at the time of developing draft guidelines
- OPTIONAL — approach accepted by Experts as having the highest diagnostic sensitivity in detecting histologic HSIL (CIN2+) at the time of developing draft guidelines

Dedicated obligatory biopsy types are recommended for all protocols, which does not exclude individualization of the decision to taking biopsy from other colposcopy suspected areas, which is depending on the clinical situation.

### BASIC PROTOCOL — minimal colposcopy approach

According to the main goal of the guidelines a minimal colposcopy scope is recommended — the basic protocol should therefore be treated as an obligatory minimum colposcopy approach, which includes:

- ECC (minimum) and/or ECB (optional) in the case of:
  - TZ3 (obligatory) and TZ2 (optional) (VI-A)
  - positive status of HRHPV 16 and/or 18 (VI-B)
  - ASC-H+ (ASC-H and higher) cytologic results (VI-A)
  - positive p16/Ki67 test result (VI-B)
  - abnormal colposcopic findings or suspicious for invasion (VI-A)
  - all major screening abnormalities of precolposcopic stage when any colposcopic abnormalities were found (VI-B)
  - considering the subsequent ablation treatment (cryo- or laser ablation) (VI-A)

  [1, 3, 13, 16, 25, 29–33, 35, 44, 75, 76].
- Targeted biopsy (in particular, from lesions assessed as abnormal colposcopic findings, suspicious for invasion, suspicious metaplasia and from other suspected areas) (VI-A)

**Optional basic protocol** is also acceptable (a variant without cases listed above in the basic protocol for ECC/ECB sampling):

- always ECC (minimum) and/or ECB (optional) (VI-B)
- targeted biopsy (in particular, from lesions assessed as abnormal colposcopic findings, suspicious for invasion, suspicious metaplasia and from other suspected areas) (VI-A)

### OPTIMAL PROTOCOL — recommended colposcopy approach

**Optimal protocol** is recommended as the optimal balance between diagnostic value and the procedure extent at the time of developing draft guidelines. It includes:

- always ECC and/or ECB (VI-B)
- targeted biopsy (in particular, from lesions assessed as abnormal colposcopic findings, suspicious for invasion, suspicious metaplasia and from other suspected areas) (VI-A)
- random biopsy for major screening abnormalities if no abnormal colposcopic findings were presented, if a new SCJ is visible (biopsies from each normal quadrant as 2, 4, 8 and 10 clock position at new SCJ) (VI-B)

### OPTIONAL PROTOCOL — accepted colposcopy approach

Optional protocol was approved for the use in Polish conditions as having potentially the highest diagnostic sensitivity in detecting histological HSIL, at the time of developing draft guidelines. It includes:

- ECC and/or ECB in each case (VI-B)
- targeted biopsy (in particular, from lesions assessed as abnormal colposcopic findings, suspicious for invasion, suspicious metaplasia and from other suspected areas) (VI-A)
- random biopsy in each case of visualization of new SCJ from each normal quadrant as 2, 4, 8 and 10 clock position at new SCJ) (VI-C).

### Documentation of colposcopy

Documentation of colposcopic findings is recommended according to the IFCPC 2011. Colposcopic images should be saved in electronic medical records.

Description of the size and location of the lesion is recommended, and it covers as follows:

1. size of the lesion as number of cervical quadrants the lesion covers,
2. size of the lesion as percentage of cervix involvement,
3. location by clock position,
4. location of the lesion in relation to the transformation zone (inside or outside).

A sample colposcopy report will be presented by the Committee after completing work of all WGs.

## SUMMARY

Recognizing the need of national implementation of the consistent colposcopy practice standards with its systemic algorithmization, the comprehensive guidelines for gynecologists, and other CCS specialists, were provided to increase a diagnostic value of colposcopy in current Polish conditions. Guidelines were based on strong evidence of the literature, extensive review of current international colposcopic standards and on the Committee's own experience. A variety of currently used colposcopy approaches in Poland, levels of training and experience of colposcopists was considered during guidelines development as well.

The use of one of three following colposcopic protocols is recommended in the colposcopy examination:

1. **Basic protocol** — presents the minimal colposcopy approach. It covers taking targeted biopsy (in particular, from lesions assessed as abnormal colposcopic findings, suspicious for invasion, suspicious metaplasia and from other suspected areas) (VI-A) and endocervical sampling (ECC minimally and/or ECB optionally) in cases of: TZ3 (optional TZ2) (VI-A), positive HRHPV 16 and/or 18 (VI-B), ASC-H+ cytologic results (VI-A), positive p16/Ki67 test (VI-B), abnormal colposcopic findings or suspicious of invasion (VI-A), no colposcopic abnormalities found in association with all major screening abnormalities of precolposcopic stage (VI-B), and in cases of planning ablation procedures (VI-A).
   Performing ECC minimally and/or ECB optionally in each case (VI-B) without specific cases listed detailly in the basic protocol is also acceptable.
2. **Optimal protocol** — presents the recommended colposcopic approach. It covers taking targeted biopsy (in particular, from lesions assessed as abnormal colposcopic findings, suspicious for invasion, suspicious metaplasia and from other suspected areas) (VI-A), taking random biopsy at the new SCJ (if visualized) from each normal quadrant as 2, 4, 8 and 10 clock position in the cases of major screening abnormalities when colposcopic abnormalities were not found (VI-B) and endocervical sampling in each case (ECC and/or ECB) (VI-B).
3. **Optional protocol** — presents the acceptable colposcopic approach. It covers taking targeted biopsy (in particular, from lesions assessed as abnormal colposcopic findings, suspicious for invasion, suspicious metaplasia and from other suspected areas) (VI-A), taking random biopsy at the new SCJ in each case of its visualization from each normal quadrant as 2, 4, 8 and 10 clock position (VI-C) and endocervical sampling in each case (ECC and/or ECB) (VI-B).

### *Conflict of interest*

Professor Robert Jach reported honoraria from Gedeon Richter for lectures. No other disclosures were reported.

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