Labeling of microvessel density, lymphatic vessel density and potential role of proangiogenic and lymphangiogenic factors as a predictive/prognostic factors after radiotherapy in patients with cervical cancer

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Summary

Introduction: Angiogenesis, formation of a new blood vessel from the existing vascular network, is essential for tumor growth, progression and metastasis. Vascular endothelial growth factor (VEGF) has been identified to be one of the most important factors of angiogenesis. VEGF-C, a novel member of the family, is a relatively specific lymphangiogenic growth factor. It is tempting to suggest that cervical cancer is one of the most common malignancies in a woman's life. Its prognostic factors are tumor stage, lymph node status, histologic type, level of hemoglobin. However, little is known about prognostic or/and predictive significance of angiogenesis in cervical cancer. *Objective:* This prospective study is an attempt to evaluate serum VEGF-A, VEGF-C, microvessel density (MVD), and lymphatic vessel density (LMVD) in cervical cancer and the correlations with clinicopathologic features. *Material and Methods:* Blood samples were collected from 58 patients affected by FIGO I-IV stage cervical cancer, who were admitted to the Department of Oncology and Brachytherapy Collegium Medicum in Bydgoszcz of Nicolaus Copernicus University. Serum VEGF-A/VEGF-C concentrate was determined by means of a quantitative sandwich enzyme immunoassay (ELISA). All tumor samples were taken from cross section during the first brachytherapy. Then they were examined by immunohistochemical studies with podoplanin antibody and anti-CD31 antibody. The present analysis was used to evaluate MVD and LMVD. *Results:* The median serum VEGF-A was 734.76 pg/ml (range from 86.39 pg/ml - 2200.00 pg/ml), and VEGF-A was only correlated with after treatment hemoglobin concentration ($p = 0.046$, $R = –0.3450$). The median serum VEGF-C was 145.72 pg/ml (range 131.08 - 233.60 pg/ml). Serum VEGF-C levels measured in patients were associated with primary tumor size. We observed significantly higher serum VEGF-C in localized disease (FIGO I, II) in comparison to advanced tumors (232.44 pg/ml *vs* 152.45 pg/ml; $p = 0.034$). The median LMVD was 6.25 (range 3.5-10.0) and median blood vessel density was 12.5 (range 9.5-23.0). We found significantly higher lymphatic vessel density in patients with G1/G2 grade of differentiation than in those with G3 (9.93 *vs* 6.25; $p = 0.0398$). We observed a statistically significant correlation between MVD and LMVD; ($p = 0.032$). *Conclusion:* In conclusion, our study suggests that serum VEGF-A, VEGF-C, LMVD and MVD play an important role in tumor growth and progression in cervical cancer. Nonetheless, further studies are essential to explore the underlying mechanism.

Key words: Cervical cancer; Angiogenesis; VEGF; Microvessel density.

Introduction

Poland is among the European countries with one of the highest levels of cervical cancer, with as many as 3,345 new incidents and 1,819 deaths only in 2006 [1]. It affects young women with the incident rate increasing with age; the highest risk age group is 45-55 years. The most frequent histopathologic type is plano-epithelial carcinoma in keratinizing or non-keratinizing forms, accounting for around 95% of malignant cervical cancers [2]. The risk of involving lymph nodes accounts for 1-5% in FIGO Stage IA, 20% in IB and up to 45% in advanced tumors (FIGO III-IV). The choice of treatment is dependent on prognostic factors such as tumor size, patient's age and general condition, type and grade of tumor differentiation, invasion of lymph vessels or blood vessels, lymph node status and level of hemoglobin before treatment [3-5]. These results inspired a search for a new predictive factor which would help determine patients with high risk of metastasis to lymph nodes and/or relapse.

Angiogenesis is the process of the formation of a new blood vessel from the existing vascular network. It is essential for tumor growth, progression and metastasis. It plays a crucial role in many phenomena, both physiological and pathological, and it consists of many stages [6, 7]. Vascular endothelial growth factor (VEGF) participates in the increase in vascular permeability, edema, extravascular fibrin deposits, the formation of vascular malformations, angiogenesis, arteriogenesis, fibrogenesis and lymphangiogenesis [7-9]. One of its isoforms is VEGF-C, which was first described in 1996 [9], and

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increased VEGF-C expression is observed in hematological diseases and malignancies.

Lymphangiogenesis coexists with the processes of angiogenesis, for example as a result of VEGF-C stimulation, which in turn stimulates the processes of angiogenesis by the receptor VEGFR-2 and/or stimulates the processes of lymphangiogenesis by the receptor VEGFR-3. Factors inducing and inhibiting angiogenesis are known but still little is known about the processes of lymphangiogenesis [10].

This prospective study was an attempt to evaluate the influence of serum VEGF-A, VEGF-C, microvessel density (MVD) and lymphatic vessel density (LMVD) on the result of tumor treatment in women with cervical cancer, and the correlation of these factors with clinicopathologic features.

Material and Methods

Patients

The research was carried out in a group of 58 patients qualified for brachytherapy for cervical cancer. All women were patients of the Department and University Hospital of Oncology and Brachytherapy Collegium Medicum in Bydgoszcz of Nicolaus Copernicus University in Torun between May 2005 and November 2006. HDR was applied in 33 women and LDR in 25 women. Combined treatment consisting of brachytherapy with surgical removal of reproductive organs was administered in 33 patients in Stage I-IIA. In 17 women in Stage IIB, III and IV basic treatment was radiotherapy (brachytherapy + external beam radiotherapy). Six women were additionally treated systemically. Eight patients underwent only brachytherapy due to coexisting diseases or distant metastasis. In two out of these patients palliative chemotherapy was applied due to the coexistance of another tumor. The average age of the patients was 56, the youngest was 36 and the oldest 87. The first follow-up visit took place six weeks after completing the treatment, and the next were scheduled for every three months. Seven patients were lost to treatment as they never turned up for a follow-up visit. Their case histories are unknown and as such they were excluded from the analysis of their response to treatment. The period of follow-up observation ranged between two and 31 months.

Serum assay

Blood samples were collected from the basic vein on an empty stomach, once, at seven o'clock in the morning before the start of the treatment. VEGF-C and VEGF-A were determined by means of a sandwich enzyme immunoassay for quantitative detection of human antibodies (ELISA, Bender MedSystem detection kit). The blood was delivered to the Diagnosis Laboratory of the Centre of Oncology in Bydgoszcz. After a 10-minute centrifugation at 3000 RPM, the blood serum was frozen and stored at –70°C until examination. All serum VEGF-A/VEGF-C analyses were performed at the same time and in the same batch. Prior to the assay, the serum was thawed at room temperature and all reagents were prepared. The tests were performed with human VEGF-A/VEGF-C (ELISA). According to the test protocol, 58 sera of cervical cancer patients were assayed, two for each patient. The intensity of staining in the wells was measured immediately after the reaction had stopped in the spectrophotometer at wavelength lambda = 450 nm and at 600 nm as a referential length.

Intratumoral angiogenesis and lymphangiogenesis

Tumor samples from the uterine cervix affected by cancer were taken during the first brachytherapy under brief general anesthesia. Tumor samples were put into a brine-filled container and delivered to the Tumor Pathology Department in the Centre of Oncology in Bydgoszcz. Tissue fragments were prepared by means of cross-section into 4 μm strips, transferred into buffered formalin (citrate buffer) and stored in paraffin, where they remained until assaying. Xylene and 3% perhydrol solution were applied in the process of deparaffining. Next, the samples were incubated for 10 min in 0.5% pepsin solution at 37°C to expose antigene determinants. The next stage was incubation of rabbit serum with an antibody directed against podoplanin (anti-human podoplanin. Mouse monoclonal antibody; clone 4D5aE5E6BMS 1105 Bender MedSystem) coating on antigens of antibody. The samples were then washed in water and dehydrated and preserved by means of xylene and Canadian balm.

All slides were evaluated by two independent researchers by means of an optical microscope Olympus BX51 (Olympus Optical Co. Ltd., Japan). At a magnification 10x and 20x the so-called 'hot spots', areas of the highest density of the vessels, were subjectively chosen. The number of such areas was different in different slides, therefore from 1-3 hot spots were chosen and the mean was calculated. The photos were taken with a Color View 3u digital camera combined with the microscope.

Microvessel density in tissue sample

Test results included 52 slides because the samples of six patients were too small. The analyzed samples were reported as follows: in 38 there was no reaction with the antibody or a massive inflammatory component, which made the evaluation of blood vessels impossible; in three samples, a weak reaction with the antibody, and in four a reaction only in the big vessels. This precluded the identification of blood vessels in the samples and excluded them from further analysis. In each case the areas of the highest density of blood vessels were searched for. The rule to evaluate three hot spots was followed. Since in some samples there were fewer areas, the number of blood vessels in one, two or three areas was evaluated. Next, the mean of microvessel density in the tissue sample was calculated.

Lymphatic vessel density in tissue sample

Test results for lymphatic vessels also included 52 slides. In eight of the samples no stroma was observed; six samples were non-diagnostic, and in three there was no reaction, which excluded them from further analysis. In each case the areas of the highest density of blood vessels were searched for. The rule to evaluate three hot spots was followed as aforementioned.

Statistical analysis

Statistical analysis was carried out by means of computer software Statistica 6.0 (StatSoft, Inc.2001). The equality of continuous distributions with a normal distribution was calculated by means of the Kolmogorov-Smirnov test corrected by the Lilliefors test, and the equality of variances was assessed with Levene's test. Inter-group comparison of the variables whose distribution did not differ from the normal distribution was assessed by Student's t-test for independent trials. For these variables which did not satisfy the condition of the equality of variances, Student's t-test with a separate assessment of variances was carried out. Comparison of variables whose distribution did not satisfy the conditions of normality was made by means of Mann-Whitney U test for assessing independent trials.

The degree of correlation between continuous variables was measured with the Pearson linear correlation coefficient. To assess the dependence between variables in range scale and continuous variables, the analysis of regression was carried out by means of the General Regression Model (GRM) from Statistica 6.0 software package. The influence of selected parameters on progression-free survival was assessed by Cox's proportional hazards model. Variables which in Cox-regression analysis gained statistical significance were included in the initial model. The risk of affecting lymph nodes and no response were assessed by means of the logical regression model. In order to do that, the quotients of chances and the correlating 95% confidence intervals were calculated.

In all statistical analyses $p < 0.05$ was taken as the border value for probability coefficient.

Results

The research was carried out on a group of 58 cervical cancer patients. A detailed characterization is given in Table 1. There were patients in all FIGO stages: 23 women in Stage I (39.7%), 18 women in Stage II (31%), and 13 women in Stage III (22.4%). Four patients were in Stage IV cancer, extending to the neighboring organs and/or with distant metastasis. The total follow-up observation period of the 58 patients ranged from 2-31 months (the mean follow-up observation period was 9.7 months). Out of 51 women 20 (39.2%) had a relapse of the cancer, 16 (80%) had local relapse, four (20%) had metastasis, including two with another cancer diagnosed. The mean time of the relapse was 8.2 months (range 1-23 months).

Detailed data concerning the age, VEGF-A, VEGF-C concentration in blood serum MVD, LMVD and the level of hemoglobin (Hb) in blood before treatment are presented in Table 2. The median serum VEGF-C was 145.72 pg/ml (range 131.08 pg/ml to 233.60 pg/ml). Median serum VEGF-A was 734.76 pg/ml (range 86.39 pg/ml to 2200.00 pg/ml), and VEGF-A was only correlated with after treatment hemoglobin concentration (p = 0,046, R = -0.3450). Serum VEGF-A was not correlated with other parameters like tumor stage, histological grade, age, before treatment Hb concentration, or platelet count.

The dependencies between variables in the whole group were assessed by means of Pearson's analysis. Due to small specification of the antibody to staining blood vessels (MVD) and no reaction in the majority of the analyzed samples, the assessment was possible in only 13 slides. In the analysis a statistically significant correlation was only observed between MVD and LMVD in the tumor (p = 0.032).

On applying the analysis of regression no differences were found between VEGF-C concentration in blood serum and FIGO stages (p = 0.30801 R = 0.2850). Similarly, no correlation was observed between LMVD and tumor stage (p = 0.60585, R = 0.2421).

Relationship between VEGF-C concentration in blood serum, LMVD and tumor stage according to FIGO staging system

In the present study, VEGF-C concentration in blood serum and LMVD were compared in the two groups with regard to stage of the disease: in the FIGO Stage I-II group and in the FIGO Stage III-IV group. A higher level of VEGF-C in blood serum was observed in early stages of the disease in comparison with advanced stages, where the level was lower (p = 0.0354). With regard to LMVD in the tumor no statistically significant differences were found between the two groups (Table 3).

Table 1. — *Patient characteristics*.

	Before treatment	6 weeks after treatment CR	PR	PD
FIGO Stage:	*n = 58*	*n = 37*	*n = 4*	*n = 10*
Stage I	23 (39.7%)	18 (78.2%)	0 (0%)	1 (4.3%)
Stage II	18 (31%)	13 (72.2%)	1 (5.5%)	3 (16.6%)
Stage III	13 (22.4%)	6 (46.1%)	3 (23%)	3 (23%)
Stage IV	4 (6.9%)	0 (0%)	0 (0%)	3 (75%)
Histological grade:	n = 31	n = 19	n = 2	n = 5
G1	2 (6%)	2 (100%)	0 (0%)	0 (0%)
G2	21 (68%)	13 (61.9%)	2 (9.5%)	4 (19%)
G3	8 (26%)	4 (50%)	0 (0%)	1 (12,5%)
Treatment:	*n = 58*	*n = 36*	*n = 4*	*n = 11*
Brachytherapy + Operation	33 (56.8%)	24 (72.7%)	0 (0%)	3 (9%)
Brachytherapy + External Beam Therapy	17 (29.3%)	9 (52.9%)	3 (17.6%)	5 (29.4%)
Brachytherapy alone	8 (13.7%)	3 (37.5%)	1 (12.5%)	3 (37.5%)
Lymph node status after operation:	*n = 33*	*n = 23*	*n = 0*	*n = 3*
N0 - 19	19 (57.7%)	13 (68.4%)	0 (0%)	0 (0%)
N(+) - 12	12 (36.3%)	9 (75%)	0 (0%)	3 (25%)
Nx - 2	2 (6.1%)	1 (50%)	0 (0%)	0 (%)

CR: Complete remission; PR: Partial remission; PD: Progression.

Table 2. — *Differences in levels of circulating VEGF-C, MVD, LMVD and clinicopathological parameters in patients with cervical cancer*.

Variable	N	Mean	±	SD
Age (36-87 years)	58	56.12	±	13.43
Hemoglobin levels before treatment (g/dl)	58	12.14	±	1.84
Serum VEGF-C (pg/ml)	55	209.7	±	165.82
Serum VEGF-A (pg/ml)	37	848.46	±	568.06
Lymphatic vessel density (LMVD)	34	8.16	±	6.30
Microvessel density (MVD)	13	16.68	±	8.86

Table 3. — *Correlation of VEGF-C and LMVD with FIGO stage in patients with cervical cancer*.

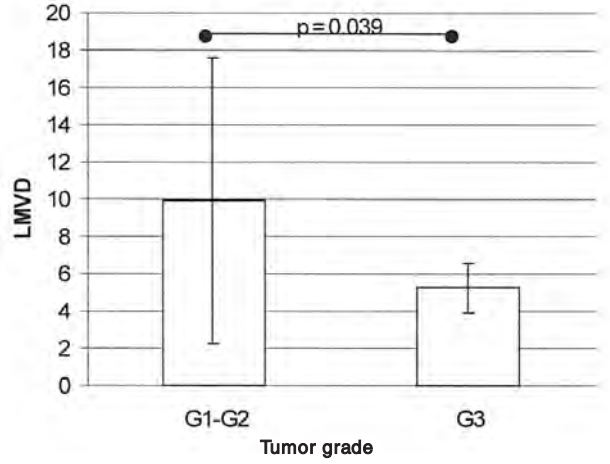
		Serum VEGF-C pg/ml			Lymphatic vessel density		
Variable		N	Mean ± SD	*p* value	N	Mean ± SD	*p* value
FIGO stage	I, II	33	**232.44 ± 188.21**	**0.0354**	21	8.95 ± 6.70	0.3280
	III, IV	13	**152.45 ± 60.63**		12	6.70 ± 7.94	

Black a value of $p < 0.05$.

VEGF-C concentration in blood serum, LMVD and the grade of tumor differentiation

The level of VEGF-C in blood serum and LMVD was compared depending on the grade of tumor differentiation in the two groups, G1-G2 vs G3 (Table 4). Higher LMVD was observed in patients with grade 1 and 2 compared to grade 3, where the number of vessels was smaller, and the result was statistically significant (p = 0.0398) (Table 4).

Table 4. — *Graph showing higher lymphatic vessel density (LMVD) in patients with grade 1 and 2 compared to grade 3.*



In terms of VEGF-C in different grades of tumor differentiation, the differences were not statistically significant (p = 0.2458).

Analysis of selected factors influencing the response to cervical cancer treatment

Higher LMVD was observed in patients with grade 1 and 2 compared to grade 3, where the numbers of vessels were smaller, and in 51 patients who reported for follow-up examination, the influence of the factors of angiogenesis, lymphangiogenesis, patients' age and the level of Hb on the result of treatment was assessed. It was measured by the grade of tumor regression, which was assessed based on clinical and diagnostic examinations. Six weeks after the termination of the treatment complete response (CR) was confirmed in 37 patients, partial response (PR) was observed in four patients, and ten patients had progression of disease (PD). During the first follow-up visit no differences were found in the aforementioned parameters in groups CR vs PR+PD.

The following examinations of the patients brought similar observations, after three, six and nine months, respectively. The only exception was the level of Hb, which in the sixth month after treatment was statistically lower in the group with PD than in the group with CR, (p = 0.0336). Due to the small sample size or no patients with a PR, the assessment of the response to treatment was impossible for the next observations.

In the next part of the investigation the risk of no response to treatment was compared between groups with: low (≤ 6.25) and high (> 6.25) lymph vessel density, low (≤ 145.7) and high (> 145.7) serum VEGF concentration, and between early (FIGO Stage I-II) and advanced disease (FIGO Stage III-IV). A crucial impact of the tumor stage on the risk of no response to treatment was observed. The analysis confirmed that the chances of recovery decreased with advanced stages of the disease (p = 0.0030).

Assessment of the risk of lymph nodes invasion in cervical cancer patients

Selected variables, such as patients' age, LMVD, MVD and the level Hb before treatment were analyzed by means of Cox's regression as potential prognostic factors for lymph node invasion. None of the parameters statistically influenced the involvement of lymph nodes.

The risk of affecting the lymph nodes was compared between groups with a low (≤ 6.25) and high (> 6.25) lymph vessel density, low (≤ 145.7) and high (> 145.7) serum VEGF levels, and between early (FIGO Stage I-II) and advanced stages of disease (FIGO III-IV). No statistically significant influence of any of the analyzed parameters on the increased risk of lymph node invasion was found. No correlation was found between LMVD and patients' age.

Discussion

In the etiopathogenesis of cancer the processes of angiogenesis and lymphangiogenesis are considered to be of crucial importance. Recently there has been an increased interest in this subject, as it is considered to be an area which raises great expectations and is of great significance. The main research trends are targeting a search for new predictive and prognostic factors. The potential of using some of these issues in developing targeted therapies are also worth noting.

Researching factors of angiogenesis and lymphangiogenesis involve a multitude of methodological and interpretative problems which have not yet been solved.

ELISA is the most commonly used method due to the ease of obtaining the pathologic material and the possibility of repeated determination of these factors.

Like any other method, ELISA is in some ways limited; for instance, secretion of VEGF in healthy cells and in tumor cells in organisms (in vivo) is unknown. Thus it is difficult to set a norm above which VEGF-C concentration is associated with the neoplastic process [10]. Observations indicate that secretion varies between different localizations of the disease and histopathologic type of tumor [11]. Determining is done with different commercial sets whose sensitivity and specificity varies, which poses difficulties in comparing results obtained by different laboratories. Moreover, we lack information as to which material is the best for determination. VEGF concentration is higher in blood serum than in plasma, which is due to the presence of blood platelets. The concentration in plasma depends on the type of the anticoagulant for blood platelet stabilization [11-13]. The obtained results frequently happen to be so diverging that they make it impossible to draw conclusions enabling researchers to work out uniform standards of determining VEGF concentration [12]. Similarly, the interpretation of VEGF concentration and other proangiogenic factors produced by the cancer, but also released by blood platelets and leucocytes in the coagulation process, is a problem which has not yet been solved [12, 14]. These interpretative difficulties are related to the fact that VEGF-C con-

centration constitutes a balance in the plasma between a free fraction of VEGF-C and a blood platelet-related VEGF-C fraction.

In scientific research it is pointed out that there is a large dispersion of the obtained results. This, according to some authors, discriminates VEGF-C as a useful marker in laboratory diagnostics [4, 15]. It is assumed that this divergence results from inflammation. From early stages of the disease, cancer is accompanied by inflammation processes with different intensity, and the activated leucocytes secrete many cytokines, including VEGF-C [16-18].

In the present study, like in most other studies, the obtained results were fairly divergent: the mean concentration of VEGF-C in blood serum was 209.7 pg/ml+/-165.82 pg/ml [17, 19-22].

A crucial and yet unsolved problem in determining VEGF-C is determining the cutoff point between low concentration connected with physiological processes ongoing in the organism and high concentration indicating cancer; especially in healthy people quite a huge difference in proangiogenic factor concentration can be observed [11, 23].

Based on multiple studies, Gasparini and other experts [12, 24] have presented international recommendations for determining the processes of angiogenesis and lymphangiogenesis. They include the guidelines for applying the antibody directed against endothelial molecule CD31, which is described to be useful for staining small and large vessels in normal tissue and in tumors. On the other hand, the study reports that this antibody has low specificity and that it does not stain lymphatic vessels [12, 24]. Despite these limitations CD31 was chosen for the study mostly due to the fact that it does not stain lymphatic vessels, which allows the determination of the real number of blood vessels in the tumor. In case of other antibodies the reaction takes place with both blood and lymphatic vessels, making the independent assessment of either vessel group impossible. Another factor which limits the usefulness of an antibody directed against CD31 is its presence in cancerous and inflamed cells. These processes are frequently connected with each other. Our observations confirm the problem. An extensive inflammatory component, expressed in the intensity of the reaction with the antibody anti-CD31 in the entire field of view, masked the presence of blood vessels and in many situations made the interpretation of the results impossible. The antibody directed against molecule CD31 is characterized by the fact that during identification of the antigen and triggering the immunoreactivity of the antigen, due to microwave activity, it may lead to lost activity, making it impossible to read the results. This happens mainly if cancer is accompanied by an extensive inflammatory component. In the analyzed material there were slides in which inflammation processes prevented the researchers from determining microvessel density (MVD). These slides were excluded from the analysis, as the objective was to search for small vessels which formed as a result of intensified angiogenic processes.

In the present study VEGF-C concentration in blood serum, LMVD and MVD were analyzed in relation to the progression of the disease according to FIGO stages. In the scientific literature there is a big divergence in obtained results. The studies by Mathur *et al*. and others indicated that the higher the levels of VEGF-C concentrations and other proangiogenic factors are the more advanced stage of the disease, which is opposite to what was observed in the present study [15, 25, 26]. Marthur *et al*. [15] studied VEGF-C content in cervical dysplasia and cervical cancer patients. They observed that VEGF-C concentration in blood serum increased with cervical dysplasia stage CIN 1,2,3, and that it was higher in cervical cancer than in cervical dysplasia patients. The concentration was highest in advanced disease and in patients with distant metastasis. However, there are multiple studies which do not confirm such relationship [4, 10, 11, 17, 27, 28]. Duff *et al*. [11] analyzed VEGF-C concentration in colorectal cancer. Astonishingly they demonstrated that the highest levels were observed in volunteers in comparison to patients, and the result was statistically significant (15.2 U/ml *vs* 10.5 U/ml). It seems to be obvious that the more advanced the stage of the disease is, the higher the concentration is of proangiogenic factors. On the other hand, the most intensified processes of new vessel formation occur in a small tumor. It is related to the change in the route of nutrition.

A part of the present study was the analysis of LMVD in tumors, depending on the stage of the disease. LMVD was not significantly different in particular stages. Similar results were obtained in the analysis of patients divided into two groups, early stages (FIGO I-II) and advanced stages (FIGO III-IV). Thiele *et al*. [29] suggest that high LMVD does not necessarily mean the presence of metastasis. According to the authors in order for this to happen the tumor must be invasive, which depends on the tumor itself, on its localization, vessel density and proangiogenic factors. Therefore, the correlation between LMVD and clinical and pathological factors may not be observed. In a study by Longatto-Filho *et al*. [30] LMVD was assessed in three groups: healthy patients, cervical dysplasia patients and cervical cancer patients. LMVD varied between the control group and the cervical dysplasia patients. Statistically significant differences were observed in women with different stages of dysplasia, as well as between the control group *vs* the dysplasia group *vs* the cancer group.

In the present study we also dealt with MVD in tumors. Apart from the correlation between MVD and LMVD, no association with other clinical or pathological parameters was observed. The obtained results should be treated with a healthy degree of scepticism. The fact that there was a restricted number of women with stained MVD was a considerable limitation of the study and had a vital impact on its results. In studies by other authors concerning MVD the results are quite diverse. The conclusions reached by different authors are contradictory. In some studies no association with clinical and pathological parameters was found, while in others MVD was associated with the stage of disease [31-36], with metastasis to lymph nodes [37] or with overall survival [33, 38].

In the next stage of the present study VEGF-C concentration in blood serum and LMVD was investigated in relation to the grade of tumor differentiation. It was demonstrated that higher levels of LMVD were associated with G1 and G2 grade, compared to G3, respectively, 9.9 vs 5.25 ($p = 0.0398$). The differences in VEGF-C content in particular groups of patients did not reach levels of statistical significance. Gao *et al*. [39] analyzed slides of 147 cervical cancer patients in FIGO Stage IA and IB. The authors confirmed the correlation between LMVD and the grade of tumor differentiation.

The next stage of the present study was to compare the level of Hb with VEGF-C concentration in blood serum, LMVD and MVD in tumors. Decrease in the level of Hb was accompanied by increase in LMVD, but the observed trend did not reach levels of statistical significance. In the published sources there have been few authors investigating the relationship between the level of Hb and the processes of angiogenesis and lymphoangiogenesis. Ferrero *et al*. [40] analyzed 72 ovarian cancer patients. They demonstrated an inverse correlation between the level of Hb and MVD in tumors. Such relationship was not confirmed by other authors dealing with this subject, like Kayaa *et al*. or Gasińska *et al*. [28, 38].

In the present study the predictive value of proangiogenic and lymphoangiogenic factors for prognosis was not confirmed. The differences in concentration of the analyzed parameters were not statistically significant between groups with CR, PR and PD. Higher LMVD and overexpression of VEGF-C in tumors correlates with metastasis to the lymph nodes and poor prognosis [10, 17, 41]. Such relationship is confirmed in esophageal, stomach, thyroid and pancreatic cancer. It makes it possible to assume that the concentration of VEGF-C circulating in the organism should be higher, although the results obtained by different authors are different [4, 10, 15, 27, 33, 38, 41]. Many data from the published literature reports indicate that LMVD in tumors may correlate with overall survival and disease-free survival rate. It is assumed that intensification of lymphoangiogenic processes may lead to tumor progression and worse prognosis. The correlation between LMVD and worse prognosis was confirmed in breast cancer, melanoma and in head and neck cancers, but it was not confirmed in oral and ovarian cancer [29, 37, 39, 41]. In the published literature the explanation of this situation is sought. It is emphasized that a key role is played by the ignorance of different mechanisms at a molecular level, small number of patients taking part in studies, and, mostly, no established methodological standards influencing the results of the conducted tests [11, 12, 14, 24]. The risk of no response to treatment in uterine cervical cancer patients is an important issue, and as such it was compared between early (Stage I-II) and advanced (Stage III-IV) of the disease according to FIGO. The risk of no response to treatment increased with the more advanced stages of the cancer ($p = 0.0030$). The results are similar to those obtained by other authors, in which tumor stage was the strongest prognostic factor in Cox's regression and multivariate Cox regression analysis [3, 34, 39, 42].

The present study did not demonstrate any influence of VEGF-C concentration in blood serum, LMVD or MVD in tumors, FIGO stage, patients' age and Hb concentration on the risk of lymph node invasion. In the published literature there have been discrepancies in the results obtained in particular laboratories. The authors emphasize that the mechanisms of this process are not fully known [11, 24, 28, 29, 39, 42]. Such correlation seems to be obvious and expected. Its existence was first confirmed by Tamura *et al*., where they demonstrated that the risk of invasion of mediastinum lymph nodes in case of non-small cell lung carcinoma increases with VEGF concentration in blood serum [43]. However, subsequent publications did not confirm this relationship [4, 11, 44]. Increased risk of metastasis to lymph nodes with high activity of proangiogenic and lymphoangiogenic factors is explained in several ways. One of the hypotheses assumes, that by means of VEGF-C activation the proliferation of lymphatic cells of endothelium is stimulated and new vessels are formed. They might induce lymph node metastasis. This hypothesis seems to be supported by the fact that newly formed lymphatic vessels are characterized by a dysfunction, which makes metastasis to lymph nodes easier [45].

Progress in the knowledge of molecular biology has initiated a multitude of studies. Yet, a practical application of the presently available information to patients' advantage in clinical data remains to be a challenge. The abovementioned research tools, new technologies and strategies may facilitate progress in the area. Nevertheless, from the perspective of personal experience this goal still seems to be far off.

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