Role of tumour- associated neutrophils in tissue material and systemic neutrophil inflammation in colorectal cancer patients

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ABSTRACT

Introduction: Colorectal cancer (CRC) is one of the most common malignant cancers worldwide. Immune response is appear to be inseparable component of each part of tumorigenesis. Moreover, several studies have shown that some populations of neutrophils, called tumor-associated neutrophils (TANs) can be also actively involved in the tumor growth, anggenesis and development of the distant metastases in various cancer tissues.

Purpose: To investigate the prognostic significance of TANs in the tumor tissue in correlation with absolute neutrophil count (ANC) and anatomoclinical features of colorectal cancer patients.

Materials and methods: We retrospectively analysed a tissue material of primary tumour mass and hematologic parameters from whole blood obtained form 160 patients diagnosed with CRC in correlation with anatomoclinical variables and disease-free survival time (DFS). Analysis of TANs in tissue material was performed by two independent pathologists under light microscopy blinded to patients' clinical information. ANC of whole blood samples was obtained within 3 days before and 7 days after the surgical treatment. We propose to exanimating of the local and systemic immune response based on the tumor- associated neutrophils in material tissue of in the invasive front and center of the primary tumor mass and ANC in whole blood samples obtained before and after surgery. Additionally, we added to mentioned above parameters the tumor progression status including the invasion of cancer cells to lymphatic vessels, lymph node involvement and the presence of distant metastasis.

Results: Combination of TANs and ANC in invasive front of tumour and in main mass of tumour were correlated with many anatomoclinical features linked with disease progression. Combined parameters of TANs, ANC and tumour progression status was associated with lymphatic invasion, lymph node involvement, TNM stage (p= 0.009), pT stage (p= 0.032) and Crohn's-like aggregates (p= 0.042). Results of centre tumour mass and ANC showed that patients with high TANs and high ANC (group 1) live longer than patients with high TANs and low ANC (group 2) (p= 0.038-3year DFS; p= 0.034- 5 year DFS).

Conclusion: TANs and ANC may have significant role in the tumour progression in colorectal cancer, but it may vary depending on the circumstances of their collection, including both tumour location and the time of cell collection.

Key words: Tumor-associated neutrophils, TANs, neutrophil count, whole blood samples, colorectal cancer

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INTRODUCTION

Colorectal cancer (CRC) is one of the most common malignant cancers worldwide [1]. Currently, patients with CRC can be treated using advanced surgical techniques, chemodrugs, and molecular-targeted drugs [2]**.** However, most of them do not respond to treatment and show progression of the disease manifested by distant metastases [3]. The formation of metastatic lesion is a multistep process that includes extensive vascularization and invasion of cancer cells into thin-walled vessels at the primary tumour site, aggregation of the transformed cancer cells in blood circulation and, finally, the extravasation of distant organ parenchyma and formation of micrometastases [4].

An immune response appears to be an inseparable component of each part of tumorigenesis. It has become evident that several types of cells, such as fibroblasts (cancer-associated fibroblasts, CAF), macrophages (tumour-associated macrophages, TAMs), and mesenchymal stem cells take part in the development of the tumour microenvironment [5,6]. It has been reported that normal host cells, such as CAFs, can be responsible for growth, invasion, and metastasis of cancer cells [7]. Moreover, TAMs occurring in the tumour microenvironment act as pro-tumour players that stimulate tumour growth and metastases, sustain angiogenesis and develop matrix remodelling [8].

Additionally, several studies have shown that some populations of neutrophils, called tumor-associated neutrophils (TANs) can be also actively involved in tumour growth, angiogenesis and development of distant metastases in various cancer tissues [9]. Physiologically, neutrophils are the first-responder of the innate immune system in response to pathogens. Recently, they have appeared to be enrolled in the regulation of innate and adaptive immune systems due to their polarization ability [10]. TANs are divided into two subpopulations based on their cancerous characteristics including surface markers, transcriptional regulators and cytokine profiles: the anti-tumorigenic ‘’N1’’ and the pro-tumorigenic ‘’N2” [11]. Neutrophils are able to secrete various inflammatory cytokines, chemokines and angiogenic factors. It has been proved that “N1” neutrophils are characterized by evaluated cytotoxicity and reduced immune-suppressive property [12]. They produce tumour necrosis factor (TNF)-α, intercellular adhesion molecule (ICAM)-1, reactive oxygen species (ROS), and the First apoptosis signal (Fas). Unlike N1, neutrophils type N2 act as a stimulator of tumour growth by expressing arginase, matrix metalloproteinase-9 (MMP-9), vascular endothelial growth factor (VEGF) and other numerous chemokines [11]. In addition, it has been reported that active neutrophils can release networks composed mainly of fibres of decondensed DNA, known as neutrophil extracellular traps (NETs). NETs stimulate the migration and extravasation of cancer cells [13]. Tohme et al. showed that NETs regulate the activation of mitogen-activated protein kinase (MAPK) signaling and cause cellular growth, migration, and invasion of colorectal cancer cells [14]. Moreover, TANs secrete the extracellular matrix-degrading protein, such as MMP9 that promotes microenvironment remodelling and angiogenesis [15]. Therefore, the aim of the study was to evaluate TANs in the tumour tissue in correlation with ANC and anatomoclinical features of colorectal cancer patients.

MATERIALS AND METHODS

Materials

Patients in the study group were operated on the Surgical Oncology Department, Comprehensive Cancer Centre (Bialystok, Poland) from April 2014 to December 2016. Clinical data included age, sex, tumour size, tumour growth, location and metastasis. The patients were diagnosed before surgery by electrocardiography, spirometry, X-ray and computerized chest tomography. In addition, the entire group underwent routine laboratory tests such as blood count test, lipid profile and arterial blood gas test. The inclusion criteria were as follows: (1) pathologically confirmed colorectal cancer; (2) treatment with radical resection and (3) not having received anti-inflammatory therapy (e. g. nonsteroidal anti-inflammatory drugs- NSAIDs). The exclusion criteria were: (1) incomplete clinic-pathological and follow-up data; (2) presence of haematological disorders.

Methods

Postoperative tissues were fixed in 4% buffered formalin for 24 to 72 h at room temperature. Small sections of tissues were embedded in paraffin. Sections (4 μm-thick) were cut from paraffin blocks and stained with haematoxylin and eosin (H&E, POCH S.A.; Avantor Performance Materials Poland) according to the manufacturer's protocol. The slides were deparaffinised in an oven at 60°C for 5 min. In the next step the slides were rehydrated in xylene (three washes, 10 min each) and graded ethanol (100, 95, 85 and 75%, 1 min at each concentration). The microscopic examination of tissues was performed by two pathologists. The pathologists determined histopathological features such as the type of tumour growth, tumour size, histological type, percentage of mucinous components, grade of malignancy and TNM classification (T: primary tumour, N: lymph node, M: distant metastasis or metastasis) [16]. Furthermore, they assessed invasion of cancer cells in veins, lymphatic vessels and nerves. Rated features of lymph node invasion include the number

of resected and invaded lymph nodes, the presence of micro- and macro-metastases, invasion of the pouch lymph node, presence of distant metastases and the size of metastases. The presence, number and size of the cancer cell deposits were also assessed [17]. Crohn's-like aggregates of lymphocyte (CRL) were performed on the basis of Väyrynen *et al.* criteria [18].

Examination of TANs in tissue material

The analysis of tumour-infiltrating neutrophils (TANs) was described in our previous study [19].

Briefly, tissue samples obtained from routine histopathological diagnosis were stained with H&E, and used to assess inflammatory cells at the invasive front and centre of the tumour with light microscopy under a high-power magnification (×400; Leica DM6 B, KAWA.SKA, Sp. z o.o., Piaseczno, Poland).

The analysis was performed by two independent pathologists blinded to patients' clinical information. Morphologically, neutrophils are polymorphonuclear cells with segmented nuclei that present clumped chromatin, eosinophilic cytoplasm and pink granules. The cells were counted multiple tumour sections (4-5 slides) and quantified as an average percentage of all the cells examined. Neutrophils were divided into two groups: ''low'' (0-20% neutrophils of all types of inflammatory cells) and ''high'' (more than 21% neutrophils).

Blood samples examination

Blood samples were obtained within 3 days before and 7 days after the surgical treatment. Venous blood samples were also obtained from 42 healthy controls (female-21, male-21; mean age 45 years old; min-max 25-65 years old). The differential white blood cell count was determined using an XN-1000 automated haematology analyser (Sysmex Co., Kobe, Japan).

Normal total neutrophil count ranges between 1.55 and 6.78 $\times 10^3$ cells/μL (mean 3.75 $\times 10^3$ cells/μL).

The total neutrophil count in colorectal cancer patients ranges from 1.54 to 8.63 $\times 10^3$ cells/μL (mean 6.93$\times 10^3$ cells/μL).

Carcinoembryonic antigen (CEA) ranges from 0 to 3.5 ng/ml. Carbohydrate antigen 19-9 (CA19-9) level amounts to 0-27.0 U/ml.

New parameters of combined TANs and ANC

We propose to assess of the local and systemic immune response based on the tumour-associated neutrophils in tissue material of the invasive front and centre of the primary tumour mass obtained only in the surgery and the absolute neutrophil count in whole blood samples obtained before and after surgery from patients with CRC. The classification of immune response system included the combination of 4 parameters divided into 4 groups:

1) tumour- associated neutrophils in the invasive front and absolute neutrophil count before surgery (TANs invasive front/ before ANC);
2) tumour- associated neutrophils in the centre of tumor mass and absolute neutrophil count before surgery (TANs centre/ before ANC);
3) tumour- associated neutrophils in the invasive front and ANC after surgery (TANs invasive front/ after ANC),
4) tumour- associated neutrophils in the and centre of tumor mass and absolute neutrophil count after surgery (TANs centre/ before ANC).

All groups included 4 subgroups:

1) high tumor- associated neutrophils (moderate or strong stromal neutrophils) and high absolute neutrophil count (≥ 6.80 x $\times 10^3$ cells/μL) (high TANs/high ANC);
2) high tumour- associated neutrophils (moderate or strong stromal neutrophils) and low absolute neutrophil count (≤ 1.90 x $\times 10^3$ cells/μL) (high TANs/low ANC;
3) low tumour- associated neutrophils (weak stromal neutrophils) and high absolute neutrophil count (≥ 6.80 x $\times 10^3$ cells/μL) (lowTANs/ high ANC);
4) low tumour- associated neutrophils (weak stromal neutrophils) and low absolute neutrophil count (≤ 1.90 x $\times 10^3$ cells/μL) (low TANs/ low ANC).

In the present study, we also examined the combination of TANs, ANC and tumour progression status. Tumour progression status was determined on the basis of 3 parameters, including the invasion of cancer cells to lymphatic vessels, lymph node involvement and the presence of distant metastasis. Tumor progression status was divided into 3 groups:

1) patients without invasion of cancer cells in any location (LV-/N-/M-);
2) patients with the invasion of cancer cells to lymphatic vessels and local lymph nodes, but without distant metastasis (LV+/N+/M-);
3) patients with the invasion of cancer cells to all mentioned above structures (LV+/N+/M+).

We analysed local and systemic response of neutrophils before and after surgical treatment. We divided patients into 4 groups:

1) negative status of tumour progression, high TANs at the invasive front and in the centre of primary tumor mass and low or high absolute neutrophil count (LV-/N-/M-/ high TANs centre or invasive front/ low or high ANC);

2) negative status of tumour progression, low TANs at the invasive front and in the center of primary tumour mass and low or high absolute neutrophil count (LV-/N-/M-/ low TANs centre or invasive front/ low or high ANC);
3) positive status of tumour progression (group 2 or 3), high TANs at the invasive front and in the centre of primary tumour mass and low or high absolute neutrophil count (LV+/N+/M+/ high TANs centre or invasive front/ low or high ANC;
4) positive status of tumour progression (group 2 or 3), low TANs at the invasive front and in the centre of primary tumour mass and low or high absolute neutrophil count (LV+/N+/M+/ low TANs centre or invasive front/ low or high ANC).

Follow-up data

The follow-up periods were from 2 to 5 years after the operation.

All patients underwent a detailed physical examination.

They were monitored by colonoscopy or/and radiological imaging such as computed tomography (CT) of the chest, abdominal cavity, and pelvis, bone scan, and positron-emission tomography (PET).

Furthermore, control tests were based on measurement of carcinoembryonic antigen (CEA) and carbohydrate antigen (CA19-9) levels.

All these techniques served to confirm local and distant recurrences.

Local recurrence was defined as pathologic spreading of tumours in the region of anastomosis, whereas distant recurrence was observed in liver, lungs, bones or brain.

Statistical analysis

Statistical analyses were performed using the STATISTICA 13.0 program (Statsoft, Poland).

Comparisons between the groups were analysed using the independent-samples t test, and comparisons within the groups were assessed using the paired t test.

Enumeration data were analysed using the $\chi 2$ test. Disease-free survival was defined as the time from the diagnosis of CRC to the first local or distant relapse.

The DFS rate was calculated using the Kaplan–Meier method.

The log-rank tests were used to compare the survival curves.

Prognostic factors were assessed using univariate and multivariate analyses (Cox proportional hazard regression model).

The P-value was set at <0.05 for all statistical analyses.

RESULTS

Patient's characteristics

The study retrospectively evaluated records of 160 patients diagnosed with CRC (96 men and 64 women). The mean age of the patients at diagnosis was 67.5 years, median age was 72 years (range from 32 to 88 years). The patients were divided into two groups: <60 years-old (40 cases) and ≥60 years-old (120 cases). The most frequent symptoms reported by the patients were abdominal pain, anaemia, rectal bleeding, constipation, diarrhoea and vomiting. Moreover, a large part of the study group was treated for hypertension, type II diabetes, osteoarthritis and coronary heart disease. The patients did not concomitantly receive inflammatory or immune-suppressive therapy. The TNM classification was used in CRC staging. The majority of patients were diagnosed with stage III colorectal cancer. Tumour locations were transverse (N=14), left-side (N=15), right-side (N=20), sigmoid (N=29) and rectum (N=82). The median size of the tumour was 3.5 cm (range from 0.3 to 8.5 cm). Seventeen patients (10.6%) had perineural invasion, 38 patients (23.8%) had lymphatic invasion and 46 patients (28.8%) had venous invasion. The lymph node metastases were present in 79 cases (30.6%). These patients received preoperative therapy: radiotherapy (N=39), chemo-therapy (N=7) and radio-chemotherapy (N=7). Preoperative pelvic radiation therapy amounted to 25 Gy (a total dose) in fractions of 5 Gy. The response to preoperative therapy was estimated according to the Response Evaluation Criteria in Solid Tumours [20]. Stable Disease (SD) was observed in 26 patients while Partial Response (PR) had 27 patients. Median of 3- and 5-year disease-free survival time was 11.6 and 27.6 months, respectively.

Combined parameters of TANs and ANC in correlation with clinico-pathological parameters of CRC patients

Distribution of the combined parameters of tumour- associated neutrophils, ANC was shown in Table 1. Moreover, as shown in Tables 2 TANs and ANC at the tumour invasive front were significantly associated with the number of resected lymph nodes (before surgery, $p= 0.021$ and after surgery, $p= 0.007$). In the main mass of tumour we observed a correlation between TANs and ANC, number of invaded lymph nodes and lymph node pouch invasion ($p= 0.011$; $p= 0.001$, respectively). CA19-9 after surgery was correlated with TANs both at the invasive front ($p= 0.022$) and in the main mass of tumour ($p= 0.025$). These data refer to parameters before surgery. Moreover, TANs and ANC after surgery were associated with Crohn's-like aggregates (at the invasive front, $p<0.001$, in the main mass $p= 0.003$).

Table 1. Distribution of the combined parameters of tumour- associated neutrophils and absolute neutrophils count.

Parameter N=(160)	**Before surgery**				**After surgery**				**P value**
Groups	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**	
TANs at the invasive front and ANC	44	69	16	31	20	16	12	112	**<0.001**
TANs in the main mass of primary tumour and ANC	51	63	13	33	29	14	83	34	**<0.001**

Table 2. Correlations between combined parameters of TANs and ANC before surgery, and clincopathological features of patients with CRC.

Parameter		**N**	**TANs and ANC**			
			Before surgery (p-value)		**After surgery** (p-value)	
		160	**Invasive front**	**Main mass**	**Invasive front**	**Main mass**
Age	<60 >60	40 120	0.716	0.989	0.991	0.954
Gender	Female Male	64 96	0.876	0.684	0.573	0.495
Location	Right-side Transverse Left-side Sigmoid Rectum	20 14 15 29 82	0.218	0.098	0.324	0.509
Tumour growth	Expanding Infiltrate	133 27	0.679	0.922	0.751	0.719
Tumour size	<2.5cm 2.5-5.0cm >5.0cm	27 106 27	0.507	1.000	0.873	0.135
Histological type	Mucinous Adenocarcinoma	30 130	0.982	0.070	0.523	0.676
Percentage of mucinous component	10-30% 30-50%	15 15	0.996	0.163	0.399	0.861
TNM stage	1+2 3+4	73 87	0.828	0.792	0.839	0.903
Grade of malignancies	2 3	148 12	0.248	0.206	0.543	0.535
Primary tumor invasion (pT)	1+2 3+4	65 95	0.104	0.301	0.189	0.637
Venous invasion	Absent Present	113 46	0.746	0.228	0.235	0.594
Lymphatic invasion	Absent Present	121 38	0.584	0.371	0.755	0.761
Perineural invasion	Absent Present	143 17	0.303	0.144	0.760	0.808
Lymph node metastasis	Absent Present	81 79	0.128	0.240	0.267	0.898
Number of resected lymph nodes	<5 5-10 ≥10	13 29 102	**0.021**	0.571	0.899	0.299
Number of invaded lymph nodes	<5 ≥5	51 28	0.306	**0.011**	0.397	0.628
Lymph node pouch invasion	Absent Present	41 39	0.439	**0.001**	0.907	**0.007**
Distant metastasis	Absent Present	143 17	0.493	0.825	0.240	0.236

Tumour Deposits	Absent Present	133 27	0.211	0.709	0.196	0.432
Tumour budding	Absent Present	94 66	0.762	0.206	0.935	0.077
Necrosis	Absent Focal Moderate Extensive	45 61 36 18	0.367	0.674	0.386	0.232
Fibrosis	Absent Focal Moderate Extensive	11 72 43 34	0.641	0.332	0.802	0.467
Crohn's-like aggregates	Absent Present	113 42	0.107	0.534	**<0.001**	**0.003**
CEA before surgery	Normal (<3.5 ng/ml) High (≥3.5 ng/ml)	36 124	0.602	0.895	0.861	0.906
CEA after surgery	Normal (<3.5 ng/ml) High (≥3.5 ng/ml)	53 107	0.497	0.377	0.469	0.561
CA19-9 Before surgery	Normal (<27.0 U/ml) High (≥27.0 U/ml)	25 135	0.604	0.959	0.585	0.732
CA19-9 after surgery	Normal (<27.0 U/ml) High (≥27.0 U/ml)	30 130	**0.022**	**0.025**	0.919	0.748

Combined parameters of TANs, ANC and tumour progression status in correlation with clinico-pathological parameters of CRC patients

Lymph node metastasis and number of resected lymph nodes were associated with TANs and ANC, and tumour progression status before and after surgery (Table 3, 4 and 5).

Also, lymph node pouch invasion was correlated in either case (before surgery p= 0.003, after surgery p< 0.001). Furthermore, before surgery there was a significant correlation (p= 0.025) between lymphatic invasion and combined parameters. Other parameters that correlated before surgery with TANs and ANC and tumour progression status were TNM stage (p= 0.009), primary tumor invasion (pT) (p= 0.032) and Crohn's-like aggregates (p= 0.042). The data such as age or sex were not associated with the combined parameters studied both before and after surgery. TANs in the centre of tumour mass, ANC and tumour progression status was found to correlate with tumour size (p= 0.046), TNM stage (p <0.001), venous invasion (p <0.001), lymphatic invasion (p <0.001), perineural invasion (p <0.001), lymph node metastasis (p <0.001), number of invaded lymph nodes (p <0.001), lymph node pouch invasion (p <0.001), distant metastasis (p <0.001) and tumour deposits (p= 0.038).

Table 3. Distribution of the combined parameters of TANs, ANC in the whole blood and progression tumour status

Parameter	**Groups**				**P value**
N=(160)	**1**	**2**	**3**	**4**	
TANs in the invasive front, ANC and tumor progression status	32	45	51	32	0.210
TANs in the main mass of the primary tumour, ANC and tumour progression status	34	37	70	19	

Table 4. Correlations between combined parameters of TANs at the invasive front, ANC and tumour progression status, and clincopathological features of patients with CRC

Parameter		**N**	**TANs at the invasive front, ANC and tumour progression status**	
		160	**Before surgery** (p-value)	**After surgery** (p-value)
Age	<60 >60	40 120	0.857	0.858

Gender	Female Male	64 96	0.416	0.750
Location	Right-side Transverse Left-side Sigmoid Rectum	20 14 15 29 82	0.906	0.409
Tumour growth	Expanding Infiltrate	133 27	0.986	0.642
Tumour size	<2.5cm 2.5-5.0cm >5.0cm	27 106 27	0.298	0.301
Histological type	Mucinous Adenocarcinoma	30 130	0.509	0.062
Percentage of mucinous component	10-30% 30-50%	15 15	0.498	0.255
TNM stage	1+2 3+4	73 87	**0.009**	0.069
Grade of malignancies	2 3	148 12	1.000	0.297
Primary tumor invasion (pT)	1+2 3+4	65 95	**0.032**	0.070
Venous invasion	Absent Present	113 46	0.308	0.505
Lymphatic invasion	Absent Present	121 38	**0.025**	0.089
Perineural invasion	Absent Present	143 17	0.150	0.096
Lymph node metastasis	Absent Present	81 79	**0.009**	**0.026**
Number of resected lymph nodes	<5 5-10 ≥10	13 29 102	**0.035**	**0.029**
Number of invaded lymph nodes	<5 ≥5	51 28	0.083	0.056
Lymph node pouch invasion	Absent Present	41 39	**0.003**	**<0.001**
Distant metastasis	Absent Present	143 17	0.920	0.477
Tumour Deposits	Absent Present	133 27	0.855	0.817
Tumour budding	Absent Present	94 66	0.205	0.137
Necrosis	Absent Focal Moderate Extensive	45 61 36 18	0.928	0.821
Fibrosis	Absent Focal Moderate Extensive	11 72 43 34	0.859	0.253
Crohn's-like aggregates	Absent Present	113 42	**0.042**	0.079
CEA before surgery	Normal (<3.5 ng/ml) High (≥3.5 ng/ml)	36 124	0.404	0.624
CEA after surgery	Normal (<3.5 ng/ml) High (≥3.5 ng/ml)	53 107	0.603	0.292

CA19-9 Before surgery	Normal (<27.0 U/ml) High (≥27.0 U/ml)	25 135	0.294	0.400
CA19-9 after surgery	Normal (<27.0 U/ml) High (≥27.0 U/ml)	30 130	0.158	0.266

Table 5. Correlations between combined parameters of TANs in the centre of tumour mass, ANC and tumour progression status, and clincopathological features of patients with CRC

Parameter		**N**	**TANs in the centre of tumour mass, ANC and tumour progression status**	
		160	**Before surgery (p-value)**	**After surgery (p-value)**
Age	<60 >60	40 120	0.738	0.795
Gender	Female Male	64 96	0.679	0.615
Location	Right-side Transverse Left-side Sigmoid Rectum	20 14 15 29 82	0.997	0.447
Tumour growth	Expanding Infiltrate	133 27	0.490	0.054
Tumour size	<2.5cm 2.5-5.0cm >5.0cm	27 106 27	**0.046**	0.154
Histological type	Mucinous Adenocarcinoma	30 130	0.212	0.117
Percentage of mucinous component	10-30% 30-50%	15 15	0.162	0.478
TNM stage	1+2 3+4	73 87	**<0.001**	**<0.001**
Grade of malignancies	2 3	148 12	0.543	**0.007**
Primary tumor invasion (pT)	1+2 3+4	65 95		
Venous invasion	Absent Present	113 46	**<0.001**	**<0.001**
Lymphatic invasion	Absent Present	121 38	**<0.001**	**<0.001**
Perineural invasion	Absent Present	143 17	**<0.001**	0.235
Lymph node metastasis	Absent Present	81 79	**<0.001**	**<0.001**
Number of resected lymph nodes	<5 5-10 ≥10	13 29 102	0.267	**0.003**
Number of invaded lymph nodes	<5 ≥5	51 28	**<0.001**	**<0.001**
Lymph node pouch invasion	Absent Present	41 39	**<0.001**	**<0.001**
Distant metastasis	Absent Present	143 17	**<0.001**	**0.030**
Tumour Deposits	Absent Present	133 27	**0.038**	0.537
Tumour budding	Absent	94	0.514	0.202

	Present	66		
Necrosis	Absent Focal Moderate Extensive	45 61 36 18	0.119	0.316
Fibrosis	Absent Focal Moderate Extensive	11 72 43 34	0.517	0.346
Crohn's-like aggregates	Absent Present	113 42	0.669	0.895
CEA **before surgery**	Normal (<3.5 ng/ml) High (≥3.5 ng/ml)	36 124	0.588	0.595
CEA **after surgery**	Normal (<3.5 ng/ml) High (≥3.5 ng/ml)	53 107	0.202	0.549
CA19-9 **Before surgery**	Normal (<27.0 U/ml) High (≥27.0 U/ml)	25 135	0.525	0.311
CA19-9 **after surgery**	Normal (<27.0 U/ml) High (≥27.0 U/ml)	30 130	0.477	0.639

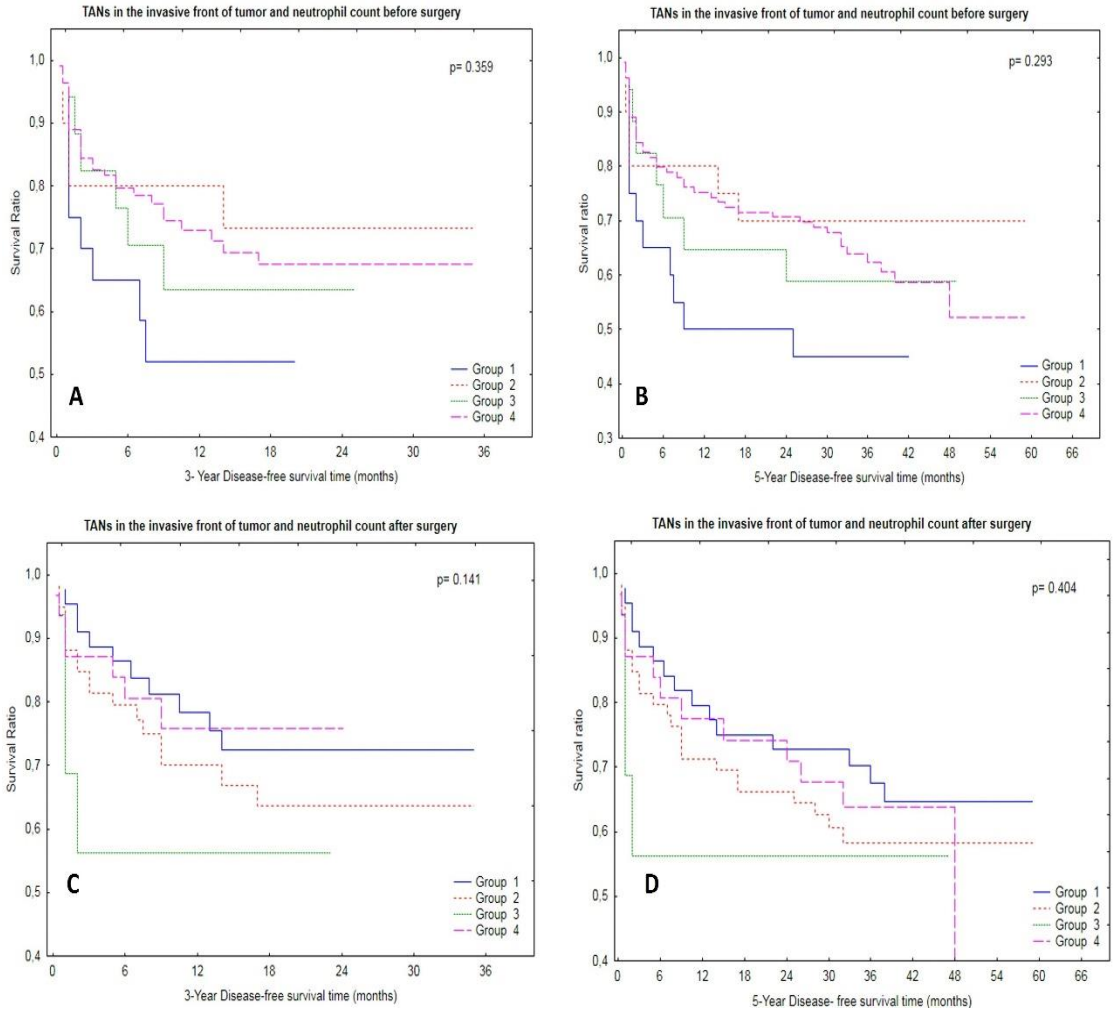

Figure 1. TANs at the invasive front of tumour and ANC obtained before and after surgery in correlation with 3- and 5 year DFS. Kaplan Meier curves for 3- year DFS for patients in TANs in the invasive front and ANC in the whole blood obtained before and after surgery (a, b). Kaplan Meier curves showing TANs in the invasive front and ANC in the whole blood obtained before and after surgery stratified according to 5- year DFS (c,d). P-value estimated with Log-Rank test. Abbreviations: Group 1- high TANs/ high ANC; Group 2- high TANs/low ANC; Group 3- low TANs/ high ANC; Group 4- low TANs/ low ANC.

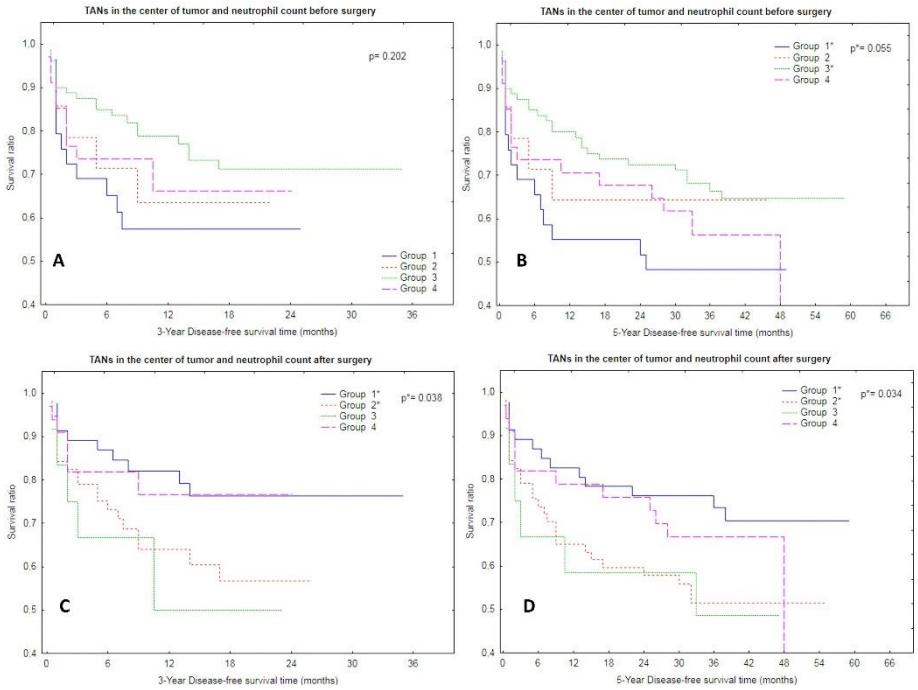

Figure 2. TANs in centre of the tumour and ANC obtained before and after surgery in correlation with 3- and 5 year DFS. Kaplan Meier analysis of 3 year DFS in CRC patients with TANs in the centre of tumour mass and ANC obtained before and after surgery (a,b). Kaplan Meier plot for 5 year DFS in patients with CRC stratified by TANs in the centre of tumour mass and ANC obtained before and after surgery (c,d). P-value estimated with Log-Rank test. Abbreviations: Group 1- high TANs/ high ANC; Group 2- high TANs/low ANC; Group 3- low TANs/ high ANC ; Group 4- low TANs/ low ANC.

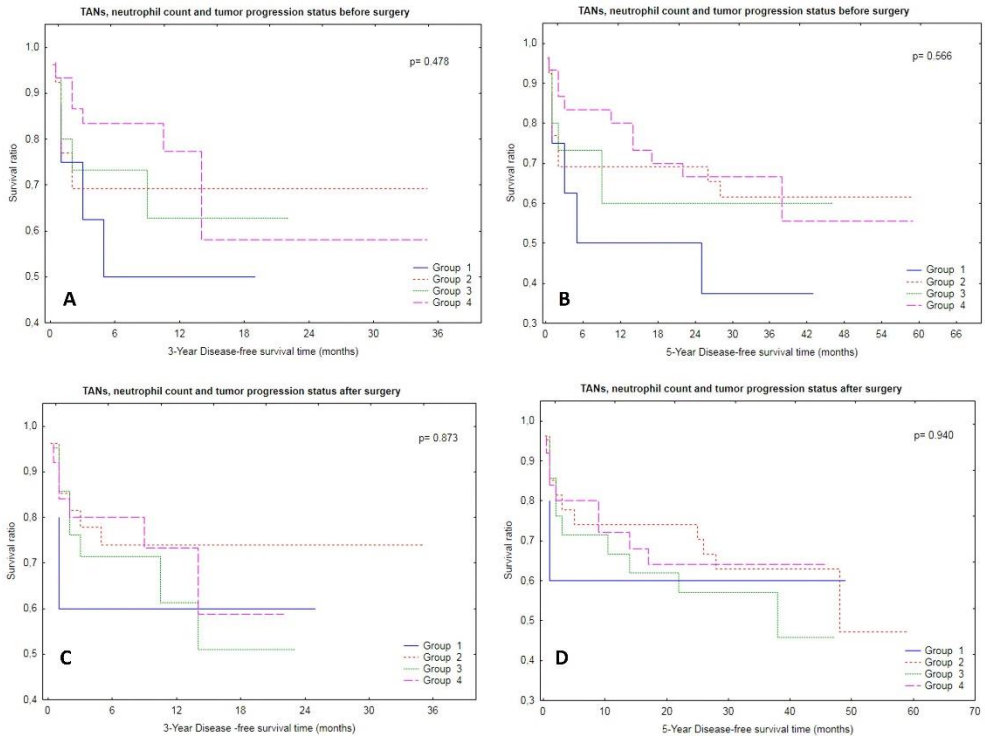

Figure 3. TANs at the invasive front of tumour mass, ANC and tumour progression status obtained before and after surgery in correlation with 3- and 5 year DFS. Kaplan Meier survival analysis of 3-year DFS according to the TANs at the invasive front of tumour mass, ANC and tumour progression status obtained before and after surgical treatment (a, b). Five- year DFS calculated by the Kaplan Meier method for TANs at the invasive front of tumour mass, ANC and tumour progression status obtained before and after medical treatment (c, d). P-value estimated with Log-Rank test. Abbreviations: Group 1- LV-/N-/M-/ high TANs at the invasive front/ low or high ANC; Group 2- LV-/N-/M-/ low TANs at the invasive front/ low or high ANC; Group 3- LV+/N+/M+/ high TANs at the invasive front/ low or high ANC; Group 4- LV+/N+/M+/ low TANs at the invasive front/ low or high ANC.

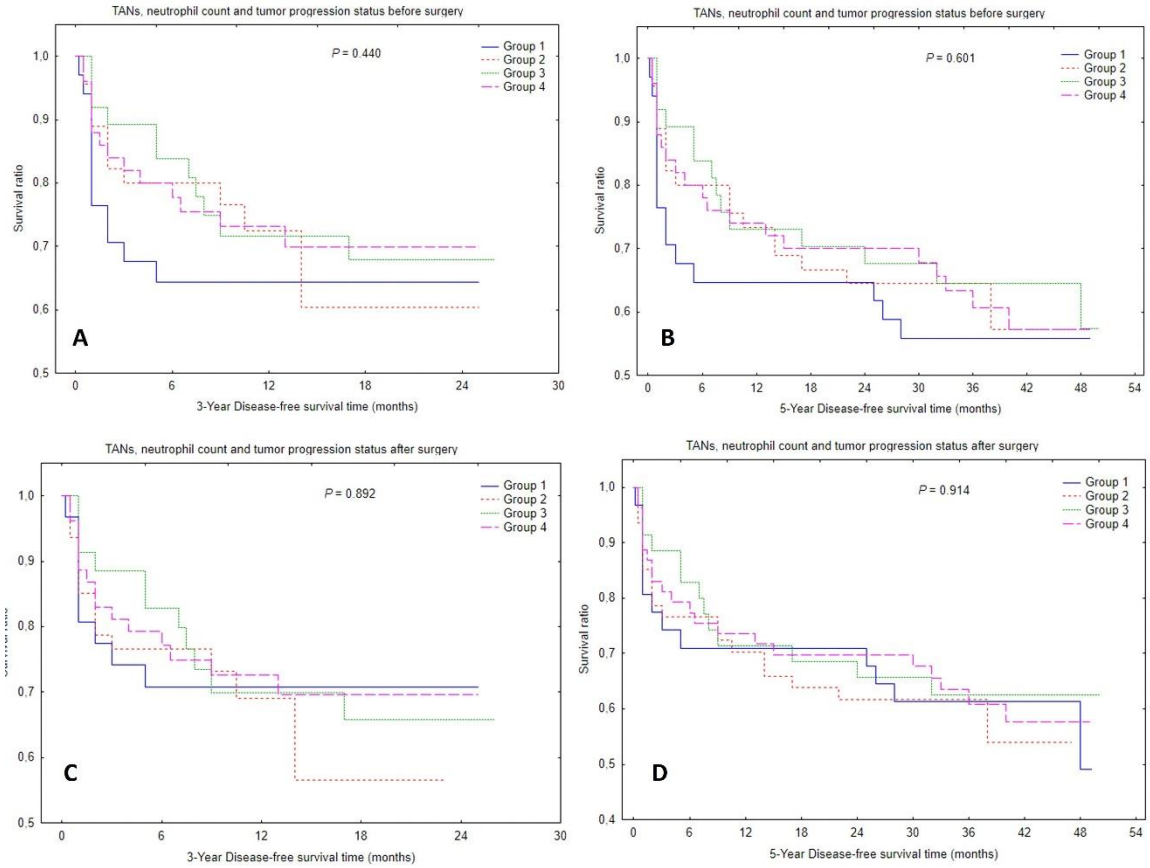

Figure 4. TANs in the centre of tumour mass, ANC and tumour progression status obtained before and after surgery in correlation with 3- and 5 year DFS. Kaplan Meier curves with 3- year DFS for TANs in the centre of tumour mass, ANC and tumour progression status obtained before and after surgery (a, b). Kaplan Meier curves displaying that the significant effect of TANs in the centre of tumour mass, ANC and tumour progression status on survival in the 5-year disease free survival time (c,d). P-value estimated with Log-Rank test. Abbreviations: Group 1- LV-/N-/M-/ high TANs at the centre of tumour mass/ low or high ANC; Group 2- LV-/N-/M-/ low TANs in the centre of tumour mass/ low or high ANC; Group 3- LV+/N+/M+/ high TANs in the centre of tumour mass/ low or high ANC; Group 4- LV+/N+/M+/ low TANs in the centre of tumour mass/ low or high ANC.

Disease-free survival of CRC and new parameters based on of neutrophil presence

Patients with group 2 and 4 characterized with TANs at the invasive front and ANC were found to have longer 3- and 5- year DFS compared to group 1 and 3. (p= 0.359- 3year DFS; p= 0.293- 5 year DFS) (Fig. 1A). On the other hand, we observed inverse results of TANs at the invasive front and ANC obtained after surgery. Only group 1 had shorter 3- and 5-year DFS, although the differences were not statistically significant (p= 0.141- 3year DFS; p= 0.404- 5 year DFS)(Fig. 1B). We also showed that group 1 patients with TANs in the centre of tumour and ANC before surgery had significantly shorter 5-year DFS as compared to group 3 where TAN count in the tumour centre was low and ANC was high (p= 0.055) (Fig. 1C). Moreover, the results of tumour centre mass and ANC after surgery showed that patients with high TANs and high ANC (group 1) live longer than patients with high TANs and low ANC (group 2) (p= 0.038-3year DFS; p= 0.034- 5 year DFS) (Fig. 2 A-D). In the present study, we also measured the relation between TANs at the invasive front and in the centre of tumour mass, ANC and tumor progression status, however, we failed to receive statistically significant differences among the groups (Fig. 3A-D and Fig. 4A-D).

DISCUSSION

In physiological condition, neutrophils are responsible for the organisation of an inflammatory response. However, circulating neutrophils are also systematically monitored in oncologic treatment due to chemotherapy-induced neutropenia [21]. Moreover, the increased number of neutrophils in the peripheral blood of cancer patients appears to be a reflection of tumour progression rather than just a consequence of the disease [22]. In the present study, we exhibited the importance of the relationship of local neutrophil infiltration in primary tumour mass with systemic absolute neutrophil count in whole blood of patients diagnosed with CRC. We indicated

that the complex of these two mentioned above parameters differed in samples obtained before and after the operation. We showed that the complex of TANs and ANC were higher in preoperative samples (groups 1 and 2) compared to those measured in post-operative materials (groups 3 and 4). We thought that the increased numbers of local and systemic neutrophils are determined by the presence of cancerous tissue rather than massive inflammation.

Circulating neutrophils and those seated in tumour tissue appear to have the ability to suppress T-cell proliferation [23]. Neutrophils located in the blood stream can release two most extensively described neutrophil-derived T-cell suppressive factors such as ROS and arginase 1 [24]. Recent studies on circulating neutrophils have revealed that the suppression of T-cell proliferation occurs through reversible cell cycle arrest, whereas in the case of the addition of l-arginine or inhibition of arginase in neutrophil/T-cell co-cultures, proliferation of T-cell is restored [24,25]. There is evidences that in the mouse model, as opposed to human neutrophils in blood stream, TANs do not release arginase 1 and accumulate it in the cytoplasm [26]. These findings are in line with our results indicating a relationship between the decreased number of neutrophils circulating in blood stream and seated in cancerous tissue after surgical treatment and the presence of Crohn's -like lymphoid reaction examined in the primary tumour mass of CRC. CLR is defined as lymphoid structures surrounding the primary tumours, excluding mucosa-associated lymphoid tissue (MALT), or pre-existing lymph nodes [18]. In addition, we pointed out that local and systemic neutrophils are associated with the level of CA 19-9 in preoperative blood samples of CRC patients. In multivariate analyses of human colorectal cancer, Zhen-yu Zhang et al. showed that preoperative circulating markers, such as neutrophil count and CA 19-9 are significantly linked with lymph node count (LNC) [27]. Briefly, lymph node count is a routine pathological parameters that is required for accurate staging of patients with CRC [28]. We also indicated the correlation between neutrophil complex and positive lymph node status, including the number of resected and invaded lymph nodes, the presence of cancer cell exceeding lymph node pouch. Recent data have demonstrated that neutrophils are able to entrap circulating tumour cells (CTCs) at metastatic sites, facilitate their extravasation and formation of metastases [29]. Moreover, Szczerba et al. demonstrated that CTC-neutrophil clusters are shown to have a highly efficient metastatic property [30].

TNM classification of CRC tumours has been a main criterion to establish patient's prognosis so far. Nowadays, there is a new classification of human CRC that has been defined on the basis of gene expression profiling [31]. Out of four consensus molecular subtypes, the ''mesenchymal'' CMS4 cluster has the worst overall and relapse-free survival. Tumours with the 'mesenchymal'' type are identified as cancerous tumour mass with high stromal infiltration with innate immune cells and fibroblasts, and high NOTCH score [32]. Additionally, research on the mouse model confirmed that the active Notch1-ICD variant (N1ICD) in intestinal epithelial cells was associated with combinations of CRC relevant inactivation of Apc or Trp53 or activation of Kras. These tumours are appear to have a high frequency of metastasis to other organs. They also show increased expression of the neutrophil chemoattractant CXCL5 and CXCR2 [33]. In the present study, we established that the complex of circulating neutrophils and those placed in the primary tumour tissue progression status-dependent manner has a higher prognostic value compared to a single, well-known and recommended factors in TNM classification. We showed that the complex of the analysed parameters was found to correlate with TNM classification, the depth of tumour invasion, the presence of lymphatic invasion, lymph node involvement and infiltration of cancerous cells outside the wall of the lymph node capsule.

Additionally, in the present study we demonstrated that patients with high TANs in the tumour centre and high ANC before surgery live shorter than patients with low TAN counts and high ANC. On the other hand, we showed that patients with high TANs and high ANC had longer 3- and 5-year DFS as compared to those with low levels of circulating neutrophils obtained after surgical operation. Our observation suggest that neutrophils have flexible ability to reorganize the immune defence against cancerous cells. The results of DFS obtained from the parameters calculated before surgery and from of tumour mass centre suggest that neutrophils of N2 type have been enrolled. Moreover, our observations including DFS time measured on the basis of neutrophils after surgery propose indicate that the N1 type of neutrophils has been involved.

Unfortunately, our study has some limitations. ANC values were obtained from patients before and after surgery, while material tissue enrolled in the present study was collected only after surgery. We have not got tissue material from colonoscopy that could be reflect to ANC before surgical treatment. In addition, we analysed neutrophils only in the basis of the morphological features and counted. However, we strongly postulate the performance of immunohistochemical staining to show the phenotypic characterization of TANs.

We concluded that TANs and ANC may have a significant role in tumour progression in colorectal cancer, although it may vary depending on

the circumstances of their collection, including both tumour location and the time of cell collection.

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Conflict-of-interest statement

The authors declare that they have no competing interests.

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