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Myeloid-derived suppressor-like cells – a potential biomarker for prognosis of colorectal cancer?

Ćelije nalik supresorskim ćelijama mijeloidnog porekla – potencijalni biomarker za prognozu kolorektalnog karcinoma?

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Abstract

Background/Aim. Myeloid-derived suppressor cells (MDSCs) represent a heterogeneous group of immature cells that have the ability to inhibit both the innate and adaptive immune response. Due to their immunosuppressive effect, MDSCs can promote the growth and progression of cancer. Colorectal cancer (CRC) is one of the most common cancers in the general population for whose advanced stages there is still no successful therapy. In addition to contributing to the development and spread of CRC, MDSCs could potentially be seen as markers of its prognosis. The aim of the study was to examine the potential prognostic role of peripheral blood MDSC counts in CRC patients. **Methods.** This prospective study analyzed the possibility of using $CD16^{low}$ granulocytes and monocytic MDSC (M-MDSC) like cells, as well as neutrophil-to-lymphocyte ratio (NLR), lymphocyte-to-monocyte ratio (LMR), $CD16^{high}/CD16^{low}$ granulocytes ratio, and monocyte-to-M-MDSC like cells ratio, before the start of the treatment, as biomarkers for overall survival (OS) in patients with CRC. The hazard ratio with the corresponding confidence interval of 95% (95% CI) was calculated to evaluate the prognostic role of MDSC in CRC. **Results.** The analysis was performed in 47 patients with stages III and IV of CRC according to the TNM/AJCC disease classification. Reliable data were obtained from 32 patients. Patient blood samples were taken before the possible start of treatment (surgery, chemotherapy). Increased percentages and absolute values of $CD16^{low}$ granulocytes, as well as absolute values of M-MDSC like cells, were associated with shorter OS ($p < 0.0066$, $p < 0.0013$, and $p < 0.0119$, respectively). The relationship between $CD16^{high}/CD16^{low}$ granulocytes ratio and monocyte/M-MDSC like cells ratio with OS indicated the existence of positive correlations between these parameters, where the higher value of this relationship indicated longer OS of patients ($p < 0.0054$ and $p < 0.0148$, respectively). The relationship between OS and NLR showed a statistically significant inverse correlation ($p = 0.0349$). No statistical significance was found in the relationship between OS and LMR. **Conclusion.** Percentages and absolute numbers of $CD16^{low}$ granulocytes, as well as the absolute numbers of M-MDSC like cells, the $CD16^{high}/CD16^{low}$ granulocytes ratio, monocyte/M-MDSC like cells ratio, and NLR ratio, may be reliable indicators of OS in patients with CRC.

Keywords:
biomarkers; colorectal neoplasms; myeloid-derived suppressor cells; prognosis; treatment outcome.

Apstrakt

Uvod/Cilj. Supresorske ćelije mijeloidnog porekla (SĆMP) predstavljaju heterogenu grupu nezrelih ćelija, koje imaju sposobnost da inhibiraju i urođeni i stečeni imunski odgovor. Zbog svog imunosupresivnog efekta one mogu da podstiču rast i progresiju karcinoma. Kolorektalni karcinom (KRK) je jedan od najčešćih karcinoma u opštoj populaciji za čije odmakle stadijume još uvek ne postoji uspešna terapija. Osim što doprinose razvoju i širenju KRK, SĆMP bi mogle potencijalno biti i markeri njegove prognoze. Cilj rada bio je da se ispita potencijalna prognostička uloga broja SĆMP periferne krvi u KRK. **Metode.** U prospektivnoj studiji analizirana je mogućnost upotrebe $CD16^{slabo+}$ granulocita i ćelija nalik monocitnim SĆMP (M-SĆMP), kao i odnosa neutrofila i limfocita (*neutrophyl-to-lymphocyte ratio* – NLR), limfocita i monocita (*lymphocyte-to-monocyte ratio* – LMR), odnosa $CD16^{jako+}/CD16^{slabo+}$ granulocita i odnosa monociti/ćelije nalik M-SĆMP, merenih pre početka tretmana, kao biomarkera za ukupno preživljavanje (UP) kod bolesnika sa KRK. U proceni prognostičke uloge SĆMP u KRK

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korišćen je parametar odnos rizika, uz odgovarajući interval poverenja od 95%. **Rezultati.** Analizirano je 47 bolesnika u III i IV stadijumu KRK, prema TNM/AJCC sistemu klasifikacije bolesti. Pouzdani podaci dobijeni su od 32 bolesnika. Uzorci krvi bolesnika bili su uzeti pre eventualnog započinjanja lečenja (operacija, hemioterapija). Pokazano je da su povišene relativne i apsolutne vrednosti $CD16^{slabo+}$ granulocita kao i apsolutne vrednosti ćelija nalik M-SĆMP bile povezane sa kraćim UP ($p < 0,0066$, $p < 0,0013$ i $p < 0,0119$, redom). Veza između odnosa $CD16^{jako+}/CD16^{slabo+}$ granulocita kao i odnosa monociti/ćelije nalik M-SĆMP i UP, ukazala je na postojanje pozitivne korelacije između tih parametara, pri čemu je viša vrednost korelacije ukazivala na duže UP bolesnika ($p < 0,0054$ i $p < 0,0148$, redom). Između UP i NLR nađena je statistički značajna inverzna korelacija ($p = 0,0349$). Nije potvrđena statistički značajna povezanost između UP i LMR. **Zaključak.** Relativne i apsolutne vrednosti $CD16^{slabo+}$ granulocita, kao i apsolutne vrednosti ćelija nalik M-SĆMP, odnos $CD16^{jako+}/CD16^{slabo+}$ granulocita, odnos monociti/ćelije nalik M-SĆMP i NLR, mogu biti pouzdani pokazatelji UP kod bolesnika sa KRK.

Ključne reči:
biomarkeri; kolorektalne neoplazme; kostna srž, ćelije, supresorske; prognoza; lečenje, ishod.

Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed cancer (after breast and lung cancer) and the second leading cause of death due to malignant disease [1]. In Europe, Hungary leads in the incidence of CRC with 51.2 cases *per* 100,000 inhabitants [2]. CRC is more common in men than women. It can develop as part of hereditary diseases such as familial adenomatous polyposis, Lynch syndrome, and Peutz-Jeghers syndrome but also as a consequence of inflammatory bowel diseases (e.g., ulcerative colitis). It most often occurs in the population as a sporadic disease, the occurrence of which is affected by older age, obesity, alcohol and cigarette consumption, excessive use of red meat and meat products, long-term use of androgens, diabetes, and even cholecystectomy [3, 4].

To better understand the nature of cancer, it is necessary to understand the complex relationships that exist within the tumor microenvironment (TME). TME is an environment composed of cells that build extracellular space and blood vessels, but also of immunologically active cells that belong to the innate and adaptive immune response, fibroblasts associated with cancer, as well as a large number of signaling molecules that are important in cancer formation, maintenance, and dissemination [5]. An important place in TME is also occupied by myeloid-derived suppressor cells (MDSCs), immature cells of myeloid origin with an immunosuppressive effect. MDSCs were first described in the 1970s in mice in which they were first phenotypically defined [6]. They were labeled as $Gr1^+CD11b^+$ cells. Two subgroups of these cells were then observed, polymorphonuclear (PMN) MDSCs (PMN-MDSCs), with surface markers CD11b and Ly6G, and monocytic (M) MDSCs (M-MDSCs), with markers CD11b and Ly6C [7]. At the same time, efforts have been made to define these cells phenotypically in humans. Due to the heterogeneity of these cells, this process is still a challenge today, and, in addition to phenotypic identification, molecular and functional determination of these cells is increasingly used. Today, these cells in humans are usually defined as $CD14^-CD15^+CD11b^+CD33^+HLA\text{-}DR^-lin^-$ and $CD11b^+CD14^-CD66b^+$, which is a phenotype shared by mature neutrophils as well and represent PMN-MDSCs and $CD14^+CD15^+CD11b^+CD33^+HLA\text{-}DR^-Lin^-$ which are similar to monocytes and represent M-MDSCs [8]. Lectin-type oxidized LDL receptor 1 (LOX 1) is increasingly mentioned in the literature as a marker of PMN-MDSCs in humans that allows better differentiation between neutrophils and PMN-MDSCs without the use of a gradient separation [9]. In recent years, another type of MDSCs has been mentioned in the human population, which is present in a small percentage. That type is called early-stage MDSCs (e-MDSCs), which lack markers for both monocyte and granulocyte populations, whose phenotype is $Lin^-HLA\text{-}DR^-CD33^+CD11b^+CD14^-CD15^-$, and contain immature progenitor and precursor cells [8, 10]. It should be emphasized that MDSCs are not cells that are exclusively located in the TME but are also present in the peripheral circulation in patients with malignant diseases. These cells can also be found in the peripheral blood (PB) of apparently healthy people as well as in many other physiological and pathological conditions unrelated to malignancy. The development and functional roles of MDSCs in various conditions, including pregnancy, inflammatory diseases of various etiologies, trauma, autoimmune diseases, heart failure and acute coronary syndrome, obesity, sepsis, Alzheimer's disease, Parkinson's disease, etc., are discussed in detail in other studies [11–20].

By now, it is well known that the circulating and tumor-infiltrating MDSCs play an important role in CRC [21, 22]. It is also accepted that the MDSCs accumulate in the late stage of malignant diseases and correlate positively with the disease progression [11]. Nevertheless, recent findings of Ma et al. [10] suggest that MDSCs accumulate even in premalignant lesions, such as colon polyposis and intraductal papillary mucinous neoplasm, and that these cells share phenotypic and functional characteristics with MDSCs seen in overt neoplasms. The development and maintenance of MDSCs in malignant diseases, including CRC, is influenced by numerous mediators released under different chronic inflammatory processes [11, 23]. These mediators encompass different chemokines and growth factors, inflammatory mediators (histamine, prostaglandins, and leukotrienes), as well as local hypoxia and low pH present within the TME [11, 24–27]. Regarding the MDSCs development in CRC, one of the particularly important factors is CCL2 which has been shown to cause MDSCs accumulation and enhance their immunosuppressive function during colorectal carcinogenesis [28]. The mechanism

of MDSCs action in CRC is also well described and relies mainly on their ability to inhibit T cell function through reactive oxygen species production and inducible nitric oxide synthase activity and to stimulate regulatory T cell development [11, 29]. These cells also promote CRC growth by releasing the exosomes containing the S100A9 protein within TME, which was recently shown for PMN-MDSCs by Wang et al. [30].

There is a considerable amount of data in the literature that connect either: the MDSCs with the clinical stage and spread of CRC disease [31]; the frequency of MDSCs in the paraffin-embedded sections with survival prediction [32]; pretreatment levels of MDSCs in the PB with progression-free survival (PFS) in patients with unresectable metastatic CRC [33] or with overall survival (OS) in patients with resectable CRC prior to surgical therapy, but without separate analysis of the two main subsets of the MDSCs [34]. In addition, the neutrophil-to-lymphocyte ratio (NLR) and lymphocyte-to-monocyte ratio (LMR) are widely used as prognostic indicators in CRC [35, 36]; however, we could not find any data regarding the prognostic value of ratios within the related cell populations such as neutrophils and PMN-MDSCs as well as monocytes and M-MDSCs in CRC patients.

The aim of our study was to examine the potential prognostic role of PB MDSC counts in CRC patients considering both main subtypes of these cells (PMN-MDSCs and M-MDSCs), as well as to analyze the prognostic usefulness of ratios within the related cell populations such as neutrophils and presumable PMN-MDSCs as well as monocytes and presumable M-MDSCs, in parallel with widely used NLR and LMR indicators in CRC patients. Given that we used fresh and lysed PB samples, in which the MDSCs gating strategy is still insufficiently defined, we designated our cells of interest as MDSC-like cells. The following text's abbreviations $CD16^{low}$ granulocytes and M-MDSC like cells refer to phenotypically matched, presumable PMN-MDSCs and M-MDSC, respectively.

Methods

Patients

The study included 47 patients diagnosed with stage III (18 patients) and stage IV (29 patients) of CRC, according to the last, eighth TNM/AJCC classification. The study was approved by the Ethics Committee of the Military Medical Academy (MMA), Belgrade (from March 10, 2016), and every patient provided a signed consent form. After the diagnosis of CRC, a blood sample was taken from the patients at the Clinic for Gastroenterology and Hepatology, MMA, Belgrade, Serbia. The patients were then monitored from June 2016 until May 2020. Patients whose eventual lethal outcome was the consequence of some other diseases or who did not report for regular follow-up examinations were excluded from the study. Sample processing was performed at the Institute for Medical Research, MMA, Belgrade. Results were obtained for 32 patients.

Samples

Three mL of venous blood were sampled from the patients with CRC. Erythrocytes were removed by lysis (EDTA, NH_4Cl, $KHCO_3$) for 20 min with constant stirring. A double wash of the nucleated cells in culture medium (RPMI640) with 5% normal human serum was performed with subsequent centrifugation and resuspension. Separation of PB mononuclear cells for comparative analysis was performed using LSM 1077 lymphocyte separation medium. Separation was performed by centrifugation at 1,200 × g for 20 min. The interphase layer was separated and washed twice in the culture medium. The number of cells was determined automatically on the Beckman Coulter ACT diff blood cell counter. The cells were resuspended at a final concentration of 1×10^6 *per* 100 μL of the suspension for further staining. After an initial comparison of the yield of the cells with the below-described phenotype between lysed samples and samples obtained with gradient centrifugation, we decided to continue the analysis with lysed samples only in order to avoid cell loss and determine the frequency and an absolute number of $CD16^{low}$ granulocytes and M-MDSC-like cells in all study participants.

Immunophenotyping of cells

The following human monoclonal antibodies were used to perform cell immunophenotyping: CD15-FITC and PECy7, CD33-PE and PECy7, CD45-ECD, HLA-DR PECy5, CD14-PECy7, CD16-FITC and PECy7, CD11b-PE, CD10-PECy7, CD3-FITC; CD19-FITC and CD56-FITC (Beckman Coulter, USA). Stained cells were analyzed on a Beckman Coulter FC 500 flow cytofluorimeter using CXP analytical software. MDSC-like cells were defined as $Lin^-(CD3^-/CD19^-/CD56^-)/HLA\text{-}DR^{-/low}CD11b^+$ cells. Polymorphonuclear and monocyte subtypes were determined based on the expression of CD14 and CD15. $CD16^{low}$ granulocytes were defined as $CD14^-CD15^+$ and M-MDSC-like cells as $CD14^+CD15^-$. The gating strategy for detection and enumeration of MDSC-like cells was based on previous work by Stanojević et al. [37]. It should be noted here that the $CD16^{low}$ granulocyte gating strategy based on CD16 expression may be a pitfall because changes in CD16 expression may also be due to changes in the functional status of healthy, mature neutrophils [38]. However, lower expression of CD16 can still be an indicator of granulocyte immaturity and/or pathological activation, and these characteristics in PB granulocytes could be attributed to PMN-MDSCs [39]. Indeed, in capecitabine-resistant CRC patients, Lu et al. [40] showed that $CD16^{low/-}$ neutrophils exerted immature gene expression profile and metabolic activity tightly related to the immunosuppressive role of MDSCs, as well as their direct immunosuppressive effects in T cell proliferation test.

Statistical analysis

All statistical analyses were performed in GraphPad Prism 9.0.2. In this study, we conducted a correlation analy-

sis to measure the level of correlation between the two variables. If the data distribution corresponded to the normal distribution, Pearson's coefficient was used to examine the degree of the linear relationship between two (numerical) variables. On the other hand, Spearman's correlation coefficient was used if the data did not have a normal distribution, was measured on an ordinal scale, or if the relationship between measured values was not linear.

Results

Immunophenotype and subsets of the MDSC-like cells in CRC patients and healthy controls

$CD16^{low}$ granulocytes and M-MDSC-like cells were identified according to the expression of CD15 and CD14, respectively, within the $HLA\text{-}DR^{-/low}CD11b^{+}CD33^{low}Lin^{-}$ population in all 47 patients in stage III and IV of the disease according to the AJCC classification. The detected immunophenotype of the targeted MDSC-like populations is given in Table 1 [37].

In brief, for detecting $CD16^{low}$ granulocytes, within the granulocyte region on side scatter (SS), cells with low and non-homogeneous expression of CD16 were gated and colored in black for tracking on other dot plots (Figure 1A) and subsequently analyzed for expression of CD11b and HLA-DR (Figure 1B), as well as for expression of CD15 (Figure 1C). On the CD45 vs. SS dot plot, black-colored cells with low and nonhomogeneous expression of CD16 showed lower levels of CD45 expression (Figure 1D) vs. eosinophils which are clearly a CD16 negative (black colored, Figure 2A) homogeneous population with strong expression of CD45 molecule (Figure 2B).

For detecting M-MDSC-like cells, the cells with positive CD11b and negative/low HLA-DR expression (Figure 3A) were checked for CD14 expression and colored black on CD14 vs. SS log dot plot for further tracking (Figure 3B). In the next step, the expression of lineage markers CD33 and CD15 was examined. The cells with $HLA\text{-}DR^{-/low}CD11b^{+}CD14^{+}CD33^{+}CD15^{-}Lin^{-}$ immunophenotype were designated and enumerated as M-MDSC-like cells. As the internal control, we used lymphocytes to compare HLA-DR (Figure 3C) and CD14 expression (Figure 3D).

Table 1

Immunophenotype of detected myeloid-derived suppressor cell (MDSC) like populations

MDSC-like subset	HLA-DR	CD3	CD10	CD11b	CD14	CD15	CD16	CD19	CD33	CD45	CD56
$CD16^{low}$ granulocytes	-/low	-	-	+	-	+	low/int	-	low	low	-
M-MDSC-like cells	-/low	-	-	+	+	-	-/low	-	low	low	-

$CD16^{low}$ granulocytes and M-MDSC-like cells were identified according to the expression of CD15 and CD14 within the $HLA\text{-}DR^{-/low}CD11b^{+}CD33^{low}Lin^{-}$ population.
M-MDSC – monocytic MDSC.
Modified Table 1 [37].

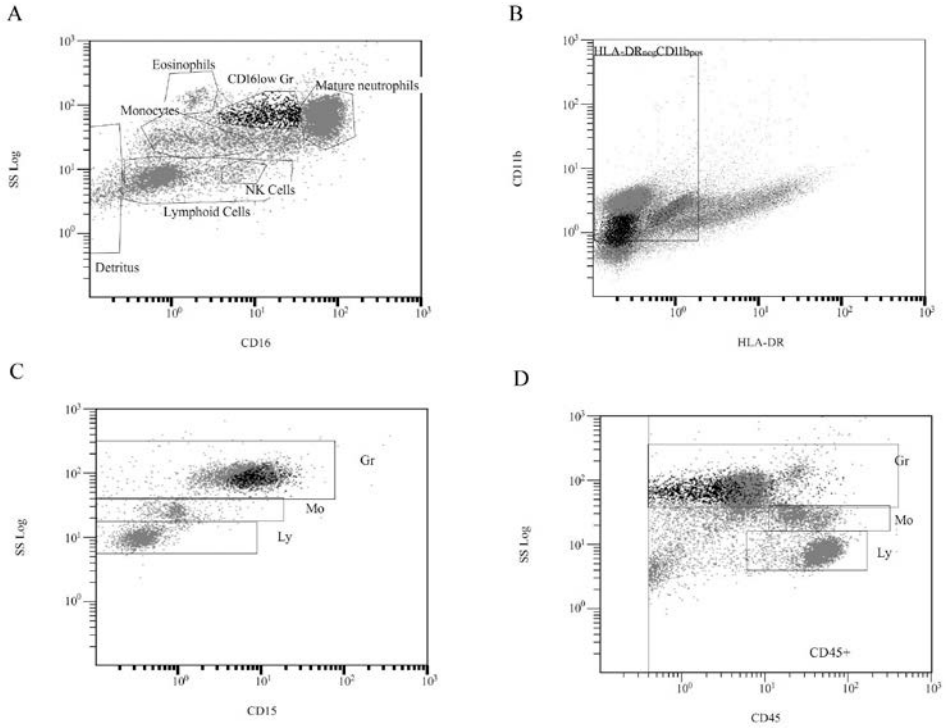

Fig. 1 – Detection of $CD16^{low}$ granulocytes (Gr) within the granulocyte region on side scatter (SS) in relation to A) CD16 expression, B) HLA-DR and CD11b expression, C) CD15 expression, and D) CD45 expression.
NK – natural killer; Mo – monocytes; Ly – lymphocytes.

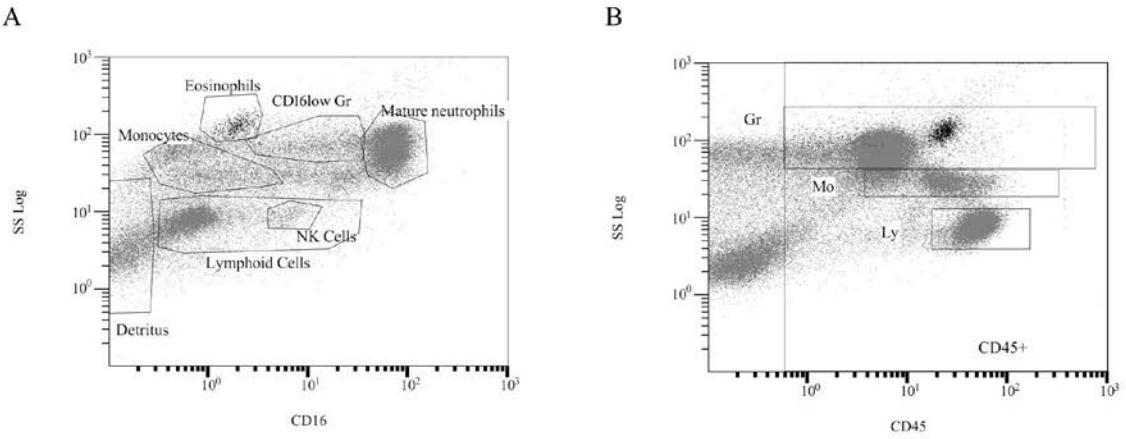

Fig. 2 –Detection of CD16^{low} granulocytes (Gr) in relation to: A) eosinophils, monocytes, natural killer (NK) cells, lymphoid cells, and mature neutrophils and to B) monocytes (Mo), lymphocytes (Ly), and granulocytes (Gr).

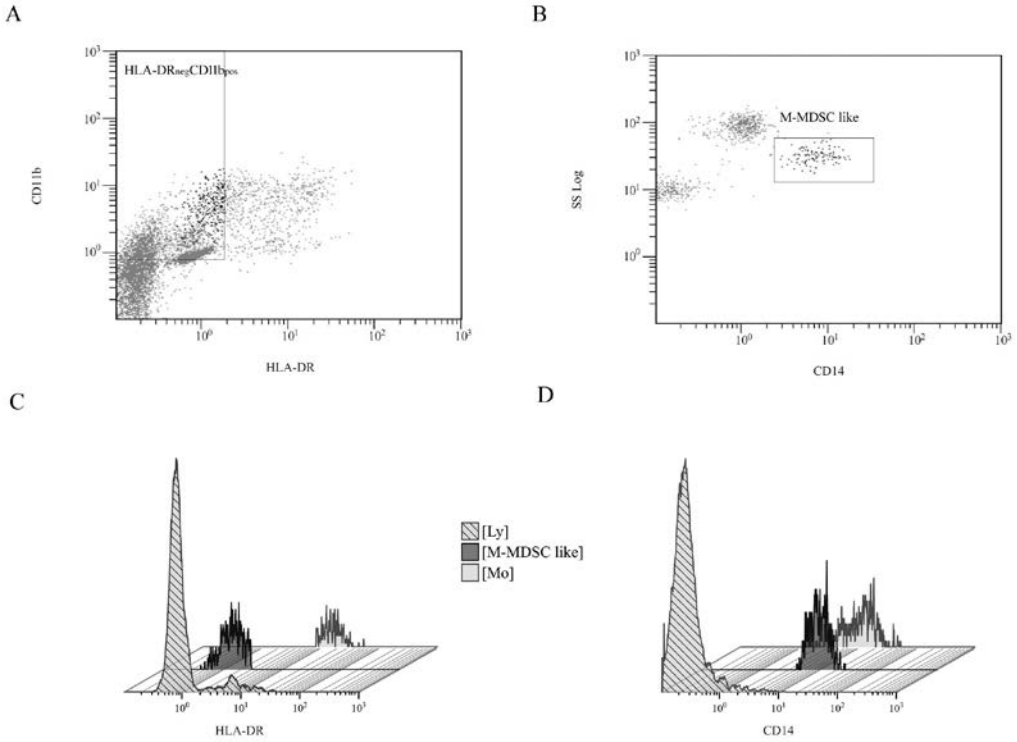

Fig. 3 – Detection of monocytic myeloid-derived suppressor cell (M-MDSC)-like cells on side scatter (SS) in relation to A) HLA-DR and CD11b expression and to B) CD14 expression. Overlay histograms show internal comparison with lymphocytes (Ly) expression of C) HLA-DR and D) CD14. Mo – monocytes.

Correlation between CD16^{low} granulocytes, M-MDSC-like cells, and overall survival

Relevant OS-related data were obtained from 32 patients. The average age of the patients was 71.9 years (men – 66.4 years, women – 77.3 years). Patients were followed for three years from the diagnosis of CRC and from taking the first sample. Spearman's correlation coefficient indicated the presence of a moderately negative linear relationship between OS measured in days and M-MDSC-like cells prevalence (Table 2). It follows from the above that patients with shorter OS had a higher percentage and an absolute number of CD16^{low} granulocytes (Spearman r = -0.4705, $p < 0.0066$ and Spearman r = -0.545, $p < 0.0013$, respectively) (Table 2) (Figure 4 A–B). For the M-MDSC-like cells, statistical significance was observed only for the absolute number of these cells (Spearman r = -0.4394, $p < 0.0119$) (Table 2) (Figure 4 C–D).

Table 2

Spearman's correlation of CD16^{low} granulocytes and M-MDSC-like cells with overall survival

	OS and CD16^{low} granulocytes		OS and M-MDSC-like cells	
	%	No/μL	%	No/μL
Spearman coefficient	-0.4705	-0.545	-0.3081	-0.4394
p-value	0.0066	0.0013	0.0863	0.0119
Significant (α = 0.05)	yes	yes	no	yes

M-MDSC – monocytic myeloid-derived suppressor cell.

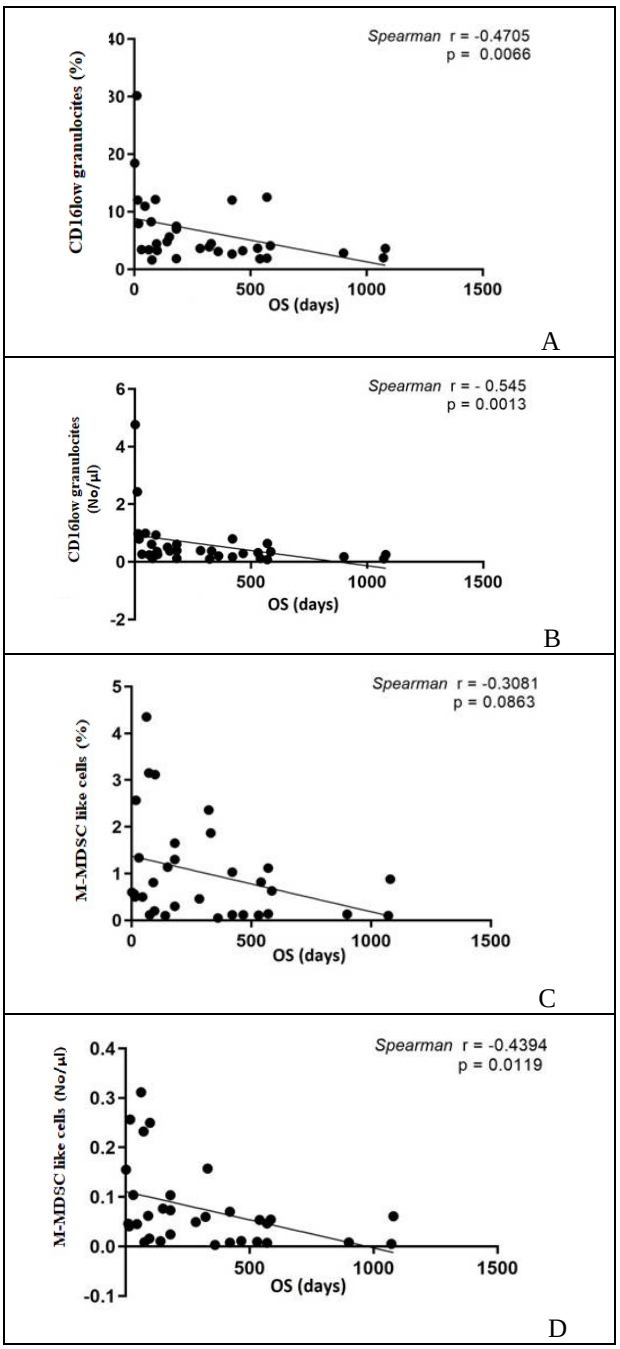

Fig. 4 – Percentages (A) and absolute numbers (B) of CD16^{low} granulocytes in relation to overall survival (OS). Percentages (C) and absolute numbers (D) of M-MDSC-like cells in relation to OS.
M-MDSC – monocytic myeloid-derived suppressor cell.

Correlation between NLR, LMR, CD16^{high}/CD16^{low} granulocytes ratio and monocyte/M-MDSC-like cells ratio and overall survival

In the same follow-up time of three years, we analyzed the relationship between OS and NLR and between OS and LMR and found a statistically significant inverse correlation with NLR (Spearman r = -0.3741, p = 0.0349) (Figure 5A). No statistical significance was found in the relationship between OS and LMR (Spearman r = 0.3328, p = 0.0627) (Figure 5B). We also tried to determine the possible connection of CD16^{high}/CD16^{low} granulocytes ratio and monocyte/M-MDSC-like cells ratio with OS. Spearman's correlation coefficient was tested first, then its significance for both ratios was calculated (Table 3). The study showed a moderately positive correlation between OS and CD16^{high}/CD16^{low} granulocytes ratio (Spearman r = 0.4802, p = 0.0054) (Figure 5C) as well as between OS and monocyte/M-MDSC-like cells ratio (Spearman r = 0.4269, p = 0.0148) (Figure 5D).

Discussion

One of the most important and common categorizations used in determining the stage and prognosis of CRC disease is the TNM/AJCC classification. However, this classification requires knowledge of the degree of invasion of the intestinal wall and lymph glands as well as the possible presence of metastases. Even though the TNM classification is the most important for assessment in clinical practice, many researchers have tried to predict the outcome of the disease or the potential response to therapy with a simpler approach and quick orientation. The NLR was initially used to assess

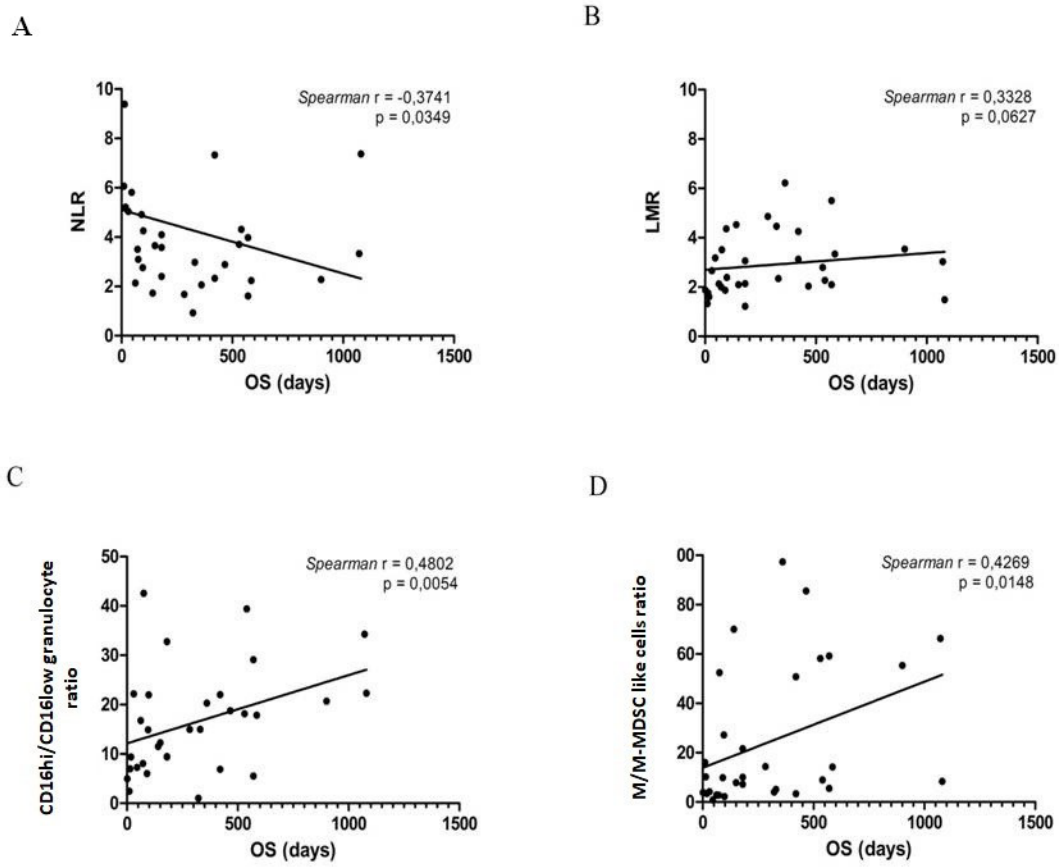

Fig. 5 – Correlation between overall survival (OS) and A) NLR, B) LMR, C) CD16^{high}/CD16^{low} granulocytes ratio, and D) M/M-MDSC-like cells ratio. M/M-MDSC – monocyte/monocytic myeloid-derived suppressor cell; NLR – neutrophil-to-lymphocyte ratio; LMR – lymphocyte-to-monocyte ratio. One data point in A) NLR is out of the y-axis range (y = 21.17, x = 1).

Table 3

Spearman's correlation between overall survival (OS) and CD16^{high}/CD16^{low} granulocytes ratio and the M/M-MDSC-like cells ratio

	OS and CD16^{high}/CD16^{low} granulocytes ratio	OS and M/M-MDSC-like cells ratio
Spearman's coefficient	0.4802	0.4269
p-value	0.0054	0.0148
Significant (α = 0.05)	yes	yes

M/M-MDSC – monocyte/monocytic myeloid-derived suppressor cell.

outcomes in patients with inflammation in the intensive care unit. Today, it often serves as a predictor of prognosis in many malignancies [35]. NLR is of special importance because it can be easily determined only from a patient's blood count sample. This method also has its limitations because the increase in this ratio does not only indicate a poor outcome for a patient who has a malignant disease but can also occur as a consequence of other diseases of the patient [41]. In recent years, both LMR and platelet-lymphocyte ratio have been used [36].

So far, in addition to these known prognostic relationships, several studies have examined the prognostic role of MDSCs in CRC patients [32–34]. The presence of circulating MDSCs in various types of solid organ cancers has been most commonly examined. Circulating MDSCs were chosen rather than the tumor-infiltrating MDSCs because they are technically easier to determine. Most patients in stage IV of the disease do not undergo surgical removal of the cancer and R0 resection but only a biopsy of the observed tumor. Likewise, further sampling and comparison of MDSC values after the tumor tissue is removed are possible only from the circulation. However, it is necessary to emphasize here that the determination of MDSCs in PB is only an indirect parameter that does not have to faithfully reflect the situation in the TME.

The association between MDSCs and the stage of disease in CRC is known [29, 42]. We tried to assess the MDSC frequencies and their absolute numbers as a potential prognostic biomarker in patients with this malignancy. A negative linear relationship between survival and MDSCs prevalence has been observed in several studies and is thought to be a consequence of their immunosuppressive function [43]. Our results confirmed that patients with shorter OS correlated positively with a higher percentage and absolute number of $CD16^{low}$ granulocytes before surgery. By M-MDSCs testing, statistical significance was observed only in the absolute number of M-MDSC-like cells. A study by Lang et al. [44], which included patients with head and neck cancer, found that patients with a high percentage of PMN-MDSCs had a significant reduction in survival and the strongest immunosuppression of T cells, while high M-MDSC values indicated a worse prognosis but not at the level of statistical significance.

Following the previously observed negative correlation demonstrated in our study, we attempted to determine whether there is an association between $CD16^{high}/CD16^{low}$ granulocytes ratio as well as monocyte/M-MDSC-like cells ratio with the OS of our patients in stage III and IV of CRC according to the TNM classification. As mentioned above, many studies dealt with NLR or LMR associations with OS in different cancer types, including CRC; however, according to the same database, we found only one study that analyzed relationships within related MDSC populations. In that study, Sheng et al. [45] found that the uncommitted MDSC/PMN-MDSCs ratio showed an inverse correlation with NLR and poorer OS outcomes in urothelial carcinoma patients with high levels of uncommitted MDSCs. In our study, we attempted to analyze relationships within related cell populations such as $CD16^{high}/CD16^{low}$ granulocytes ratio as well as monocytes and M-MDSC-like cells. In this regard, we observed that the $CD16^{high}/CD16^{low}$ granulocytes ratio and monocyte/M-MDSC-like cells ratios showed a positive correlation with OS indicating the connection between lower MDSC prevalence and better disease outcome. The reason why we decided to analyze the internal relationships within phenotypically determined related populations is that the mentioned NLR or LMR are calculated based on the results of automated blood counters that cannot recognize subtle differences between individual cells within a related population. For instance, it is generally accepted that high NLR and/or low LMR in tumor patients, including CRC patients, are associated with poor outcomes [46]. However, it is also known that the composition of circulating neutrophils in tumor patients is diverse and includes high-density neutrophils, which are thought to have anti-tumor properties and correspond to N1 neutrophils in TME, as well as low-density neutrophils with pro-tumor effects, which correspond to N2 [47]. Similarly, at least three subsets can be phenotypically identified among circulating monocytes: classical ($CD14^{high}CD16^{-}$), nonclassical ($CD14^{+}CD16^{+}$), and intermediate subset ($CD14^{low}CD16^{+}$), which may have opposite roles in tumor development, growth, and metastasis, including CRC [48]. Indeed, in our study, we found that the $CD16^{high}/CD16^{low}$ granulocytes ratio showed an even better Spearman's rank coefficient compared to NLR, while in LMR correlation analysis, the statistical significance was not achieved at all, unlike the monocyte/M-MDSC-like cells ratio which showed a statistically significant positive correlation with OS. It is known that the number of neutrophils can increase in patients with malignant disease as a consequence of chronic inflammation, as well as the relative and absolute values of MDSCs [49]. Similarly, many studies have shown that the prevalence of MDSCs increases with the stage of the disease, and the more severe stage of the disease indicates a worse prognosis and shorter survival [50–52]. The number of PMN-MDSCs and M-MDSCs in the advanced stages of the disease still increases more than the number of neutrophils and monocytes. There are at least three mechanisms responsible for the increases in the relative and absolute values of both MDSC subtypes, which include the urgent myelopoiesis in response to the existence of cancer, the plasticity of myeloid cells, and extramedullary myelopoiesis and are explained in more detail in other studies [50, 53, 54]. Regardless of the dominant mechanism, as a consequence, there is an increase in the frequency of both subtypes of MDSC, which is more pronounced in the advanced stages of the disease.

However, correlations between the relationship of $CD16^{high}/CD16^{low}$ granulocytes ratio and monocyte/M-MDSC-like cells with OS, as well as the simple prevalence of MDSCs in PB, should be viewed in a broader light of an individual patient's immune status. In their study on CRC patients, Tada et al. [33] showed that a high proportion of M-MDSCs in PB was associated with significantly shorter PFS. However, by examining other subsets of immune cells, namely $CD4^{+}$ and $CD8^{+}$ effector memory T cells (among others), they found that in patients with a high proportion of M-MDSCs, who are expected to have shorter PFS, that was

not the case if high percentages of effector memory T cells were present at the same time, and *vice versa*. The significance of more detailed immune profiling of PB cells in CRC patients was confirmed by other authors as well [55].

We consider that the main disadvantages of our study are the fact that we did not analyze repeated PB samples during the follow-up of the patients and a relatively small number of participants. Parallel analysis of lysed samples and samples obtained by gradient centrifugation should be performed to verify proper gating for $CD16^{low}$ granulocytes.

Conclusion

Our data support the potential use of M-MDSC-like cell detection and enumeration as a prognostic marker for CRC, but further research is needed. Analysis of relationships within related populations, i.e., $CD16^{high}/CD16^{low}$ granulocytes ratio and monocyte/M-MDSC-like cells ratios, have the prognostic potential and could improve the prognostic significance of other established OS indicators in CRC patients.

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