Original Paper

The level of myeloid derived-suppressor cells in peripheral blood of patients with prostate cancer after various types of therapy

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Prostate cancer is one of the most frequent cancers in men. Although several treatment options exist, their clinical effectiveness is still not satisfactory. One the possible reason of such situation might be the presence of myeloid-derived suppressor cells (MDSC) and their pro-tumorigenic activity. MDSC possess immunosuppressive ability and in many studies were shown to support tumor development and progression. In this study we addressed the question whether commonly used therapies of prostate cancer affect the level of MDSC populations in the patients' blood. We compared the level of granulocytic (Gr-MDSC), monocytic (Mo-MDSC) and early stage MDSC (eMDSC) in the blood of patients at different clinical stage and different tumor grading scores, who underwent either surgery or hormonal therapy alone or were given a combined treatment, including e.g. radiotherapy. The obtained results showed that the level of Gr-MDSC was significantly lower in all treated patients comparing to untreated group. On the other hand, surgery or hormonal therapy alone did not affect the level of Mo-MDSC. These results were independent of the PSA level, the tumor grading and clinical stage of the patients. In conclusion, we suggest that Mo-MDSC should be considered as a potential therapy target in the course of prostate cancer treatment to enhance its anti-tumor effectiveness.

Key words: prostate cancer (PC), myeloid-derived suppressor cells (MDSC), flow cytometry, prostate specific antigen (PSA).

Introduction

Prostate cancer (PC) is the second most frequently diagnosed cancer in men and the fifth leading cause of cancer death worldwide [1]. Economic development, increased consumption of animal fat, obesity and lack of physical activity have been considered as modifiable risk factors of this type of cancer [2, 3]. On the other hand, many factors including improved treatment and early detection of PC, e.g. thanks to preventive programs, reduce the death rate [2]. In the early 1990s, soon after the introduction of a prostate-specific antigen (PSA) testing to the routine diagnostic procedure, the rapid growth

of incidence of prostate cancer was observed [2, 4]. Since then, due to frequent false positive results of PSA test and severe side effects of subsequent unnecessary treatment, the usefulness of routine PSA screening is a matter of debate [5, 6]. Therefore, efforts are undertaken to discover new more reliable tests, such as urine-based, that could play a major role in a primary screening [7]. In the middle of 20th century it was noticed that prostate cancer had been influenced by androgens and could be inhibited by elimination of these hormones, either through castration or neutralization of androgen activity by estrogen injections [8]. However, despite the low level of androgens, the so called castration-resistant prostate cancer still has an ability for progression. With many possibilities for prostate cancer treatment, including watchful waiting, surgery, radio-, chemo- and biological therapy [9,10], there is still a lack of satisfactory results, as tumor resistance to the applied treatment develops quite often. Recent studies indicate that the failure of prostate cancer treatment may be due to the myeloid-derived suppressor cells (MDSC), which were shown to support tumor development and recurrence [11].

MDSC compose a heterogeneous population of myeloid cells at different stage of differentiation, which regulate the immune response [12]. Nevertheless, under chronic inflammatory conditions, infections or cancer, due to the inhibition of myeloid cell differentiation the level of MDSC is elevated [13]. Numerous studies present evidence that inflammatory factors produced by tumor may induce generation of MDSC and/or increase their activity [14]. Despite high heterogeneity, MDSC are frequently distinguished as granulocytic (Gr-MDSC) and monocytic (Mo-MDSC) [12]. Recently, a population of so called early stage MDSC (eMDSC) has also been identified [15]. In humans, minimal phenotype characteristics define Gr-MDSC as CD11b+ CD14– CD15+, while Mo-MDSC as CD11b+ CD14+ HLA-DR–/lo CD15–. Population of eMDSC is recognized as CD11b+ CD14– CD15– [16]. Both Mo-MDSC and Gr-MDSC effectively inhibit T lymphocytes activity, although using different mechanisms, whereas function of eMDSC is still not clear [16, 17, 18]. Gr-MDSC preferentially express arginase – 1 (ARG1) and NADPH oxidase. Hence, Gr-MDSC show a higher production of reactive oxygen species (ROS) when compared to Mo-MDSC, which, on the other hand, comparing to Gr-MDSC display increased expression of inducible nitric oxide synthase (NOS2) and NO production [19]. MDSC could also induce T regulatory cells, which are often correlated with cancer progression [20].

Here, we asked whether commonly used therapies of prostate cancer affect the level of MDSC populations in peripheral blood of patients.

Material and methods

Patients

Patients with PC were recruited from the Maria Skłodowska-Curie Cancer Center in Krakow. 43 PC patients (treated or untreated) and 23 healthy age-matched controls (CTR) (mean age 67.64 ±9.08 PC vs. 69.6 ±6.31 CTR; $p = 0.062$) were enrolled into the study. As the PC group was heterogeneous in respect to the treatment options, it was further divided into four following subgroups: I – patients before treatment – 21, II – patients after a surgical removal of the tumor – 7, III – patients who received combined therapy (more than one type of treatment chemo-/radio-/ hormonal therapy/ surgery) – 9 and IV – patients with hormonal therapy alone – 6. PSA level (ng/ml), the Gleason scoring and clinical staging were performed routinely. The Gleason score assigns two most common histological patterns from 1 for highly differentiated to 5 for low differentiated cancers, with summary score ranging from 2 to 10. Further characterization of the patients' group is presented in Table I, while their classification according to the American Joint Committee on Cancer (AJCC) is presented in Table II [21]. All patients provided a signed informed consent and the study protocol was approved by the local bioethics committee of the Regional Board of Medical Doctors in Krakow (no. 6/KBL/OIL/2014).

Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) were isolated from blood samples obtained from prostate cancer patients and healthy controls by standard density gradient centrifugation. Blood was collected into BD CPT™ mononuclear cell preparation tubes containing sodium citrate and Ficoll™ Hypaque™ Solution (Vacutainer System; Becton Dickinson, San Jose, CA) and the tubes were centrifuged at 1400×g for 15 min at room temperature. PBMCs were collected from the interphase, washed in PBS, resuspended in 0.5 ml of PBS and used for further analysis.

Flow cytometry analysis of Gr-MDSC, Mo-MDSC and eMDSC

For Gr-MDSC and Mo-MDSC analysis, PBMC (app. 1×10^6 cells/100 μl) were stained with following monoclonal antibodies (mAbs): anti-LIN-AF700 (anti-CD3 clone UCTHT1, anti-CD19 clone HIB19, anti-CD56 clone B159), anti-CD33– PE (clone P67,6), anti-HLA-DR-PerCp (clone L243), anti-CD11b-BV510 (clone ICR F44), anti-CD14-FITC (MϕP9), anti-CD15-PE-Cy7 (clone HI98) (BD Biosciences; San Jose, CA) for 20 min in 4°C. After

Table I. Characterisation of the patients group. The patients in the stage group were qualified according to the AJCC guidelines (Table II). The grade group depends on Gleason score (summary of the two most common histological patterns of the tumor with scores ranging from 1 for highly differentiated to 5 for low differentiated cancer)

Prostate cancer patients	All	Before treatment
	43	21
PSA level (ng/ml)	33.67 ±35.02	30.14 ±34.44
Stage groups		
2b	11	6
2c	2	2
3a	7	1
3b	17	9
4a	5	3
4b	2	1
Grade groups		
1 (6 = 3 + 3)	1	-
2 (7 = 3 + 4)	14	7
3 (7 = 4 + 3)	15	6
4 (8 = 3 + 5; 4 + 4)	7	4
5 (9 – 10 = 4 + 5; 5 + 4; 5 + 5)	6	3

incubation, cells were washed twice in PBS, suspended in 0.2 ml PBS and analyzed using FACSCanto flow cytometer (Becton Dickinson, CA, USA) and FACS-Diva software (BD). The Gr-MDSC, Mo-MDSC and eMDSC subsets were characterized as LIN- CD33+ HLA-DR– CD11b+ CD14– CD15+, LIN– CD33+ HLA-DR- CD11b+ CD14+ CD15– and LIN- CD33+ HLA-DR– CD11b+ CD14- CD15- respectively, and presented as a percent of nucleated cells (%NC) (Fig. 1). In order to determine the level of non-specific staining and cellular autofluorescence, the fluorescence minus one (FMO) control samples were prepared and analyzed in parallel.

Statistical analysis

Statistical analysis was performed using the PRISM GraphPad 5 package (GraphPad Software Inc., San Diego, CA, USA). Obtained data were analyzed using Mann Whitney test after Shapiro-Wilk normality test or one-way analysis of variance (ANOVA) with Newman-Keuls Multiple Comparison Test as post hoc test. All data are expressed as mean ± SD. $P < 0.05$ was considered statistically significant.

Results

In the present study we aimed to evaluate the level of MDSC populations in the blood of prostate cancer patients, depending on the PSA level, tumor grading, clinical stage of the patients and treatment option they received. For this purpose we used flow

Table II. Clinical classification of the patients with prostate cancer according to the AJCC guidelines [21]. The TNM staging system describes the size and extent of the primary tumor (T), metastasis to the local lymph nodes (N), and distant metastasis (M); × means any result

T	N	M	PSA (ng/ml)	Grade group	Stage group
cT1a-c	N0	M0	< 10	1	I
cT2a	N0	M0	< 10	1	I
pT2	N0	M0	< 10	1	I
cT1a-c	N0	M0	≥ 10 < 20	1	IIa
cT2a	N0	M0	≥ 10 < 20	1	IIa
pT2	N0	M0	≥ 10 < 20	1	IIa
cT2b-c	N0	M0	< 20	1	IIa
T1-2	N0	M0	< 20	2	IIb
T1-2	N0	M0	< 20	3	IIc
T1-2	N0	M0	< 20	4	IIc
T1-2	N0	M0	≥ 20	1-4	IIIa
T3-4	N0	M0	×	1-4	IIIb
Tx	N0	M0	×	5	IIIc
Tx	N1	M0	×	×	IVa
Tx	Nx	M1	×	×	IVb

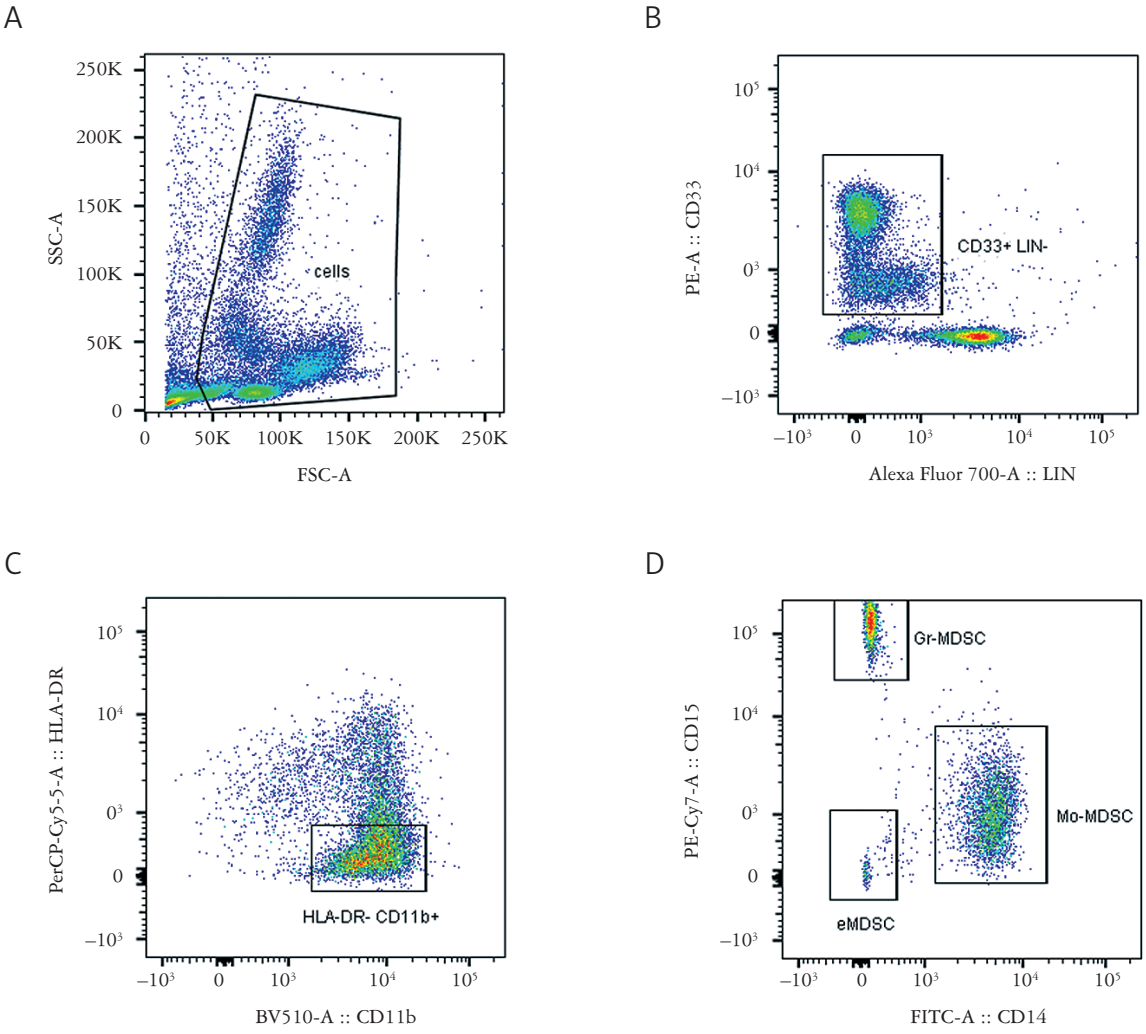

Fig. 1. Gating strategy for flow cytometry identification of Gr-MDSC, Mo-MDSC, eMDSC in peripheral blood of prostate cancer patients

cytometry analysis of peripheral blood leukocytes. The calculated mean percent value of Gr-MDSC in the whole group of PC patients was statistically higher when compared to control healthy individuals (2.53 ±2.81 in PC vs. 0.30 ±0.33 in CTR; $p < 0.0001$) (Fig. 2), as was the level of Mo-MDSC (10.67 ±8.15 in PC vs. 1.17 ±0.94 in CTR; $p < 0.0001$) (Fig. 2). In case of eMDSC, no statistically significant difference between patients and healthy controls was observed (data not shown). Although the usefulness of monitoring the PSA level is a matter of debate, it is still generally considered as a biomarker of the disease state. With this in mind, in the next step we asked if there is any correlation between the MDSC percentages and related PSA levels. We have shown such a correlation for Mo-MDSC (Pearson $r = 0.31$; $p = 0.047$) but not for Gr-MDSC (Fig. 3). In this context, it was interesting to find out whether the level of MDSC correlates with the tumor grading according to Gleason scale or clinical staging of the patients. Therefore, considering Gleason score we divided our patients into four groups (according to the AJCC classification) and compared them with the level of Mo-MDSC and Gr-MDSC in peripheral blood, respectively. The obtained results indicated that regardless of Gleason score, the prostate cancer patients presented higher level of both Gr-MDSC and Mo-MDSC populations (Fig. 4) in comparison to healthy individuals. Moreover there was no significant differences in the level of Gr-MDSC and Mo-MDSC between the groups of patients (Fig. 4). These aspects were also analyzed in respect to the clinical stage of the patients, taking into account the PSA level, TNM classification and Gleason score (grade group) (Table II) [21]. In this case, due to the small number of patients in the groups IIb and IIc, IIIa, and IIIb, and IVa and IVb they were included to group II, III and IV, respectively. In such analysis, the level of both populations of MDSC was higher

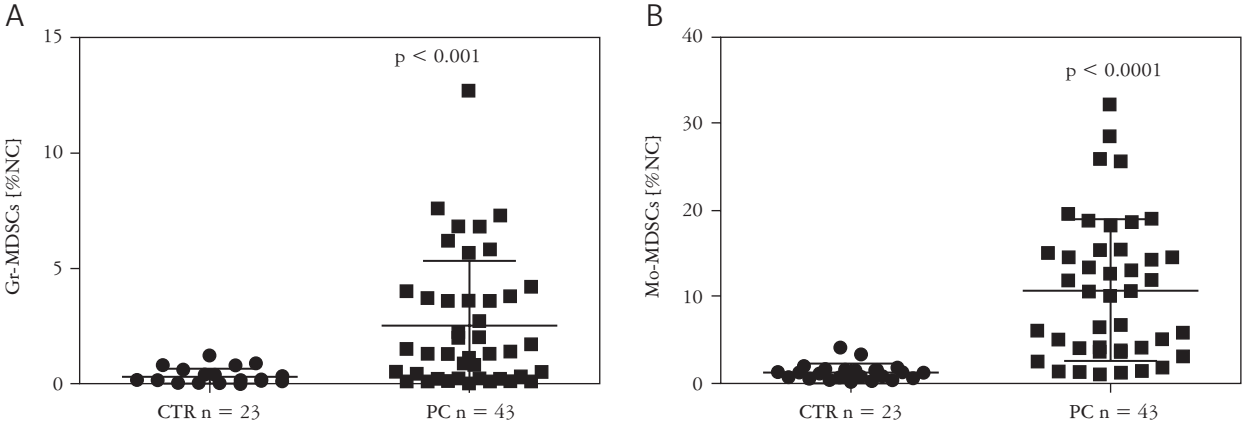

Fig. 2. Level of Gr-MDSCs (A) and Mo-MDSCs (B) in healthy control subjects and prostate cancer patients (% NC – % of nucleated cells)

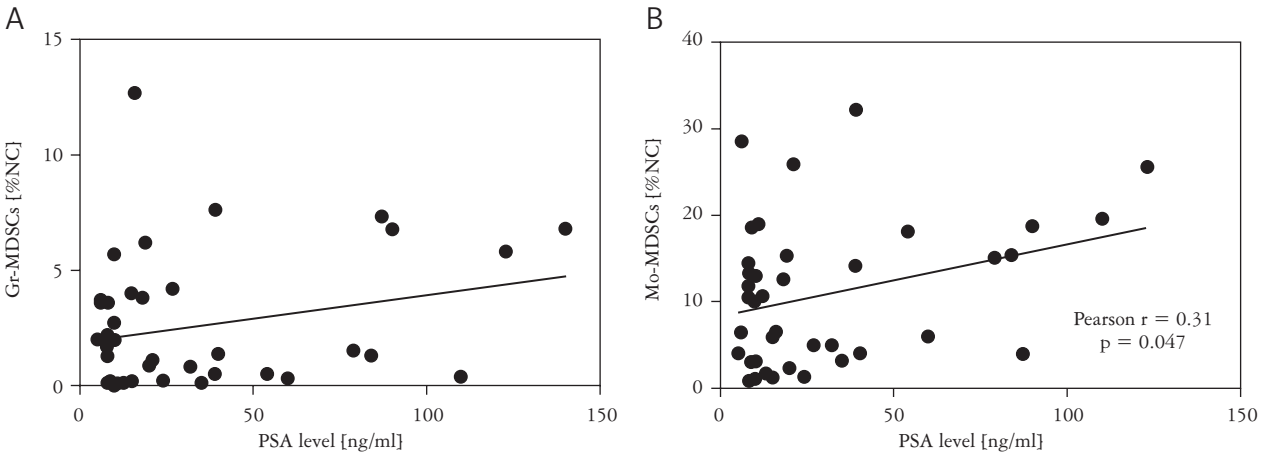

Fig. 3. Correlation between PSA level (ng/ml) and Gr-MDSC (A) and Mo-MDSC (B) percentage (% NC – % of nucleated cells)

in cancer patients in comparison to healthy controls, despite the disease stage (Fig. 5).

In the next step of analysis, a logical consequence was to evaluate the level of Gr-MDSC and Mo-MDSC in the patients' blood in respect to the mode of therapy they received. For this purpose the group of patients was subdivided into four groups, including patients before treatment, receiving surgery or hormonal therapy alone or receiving a combined therapy. The obtained data show that the mean proportion of Gr-MDSC in peripheral blood of patients was significantly higher in the group before treatment, comparing to healthy controls (3.82 ±3.23 vs. 0.30 ±0.33; $p < 0.001$) (Fig. 6), whereas the patients with formerly implemented therapy had reduced percentage of Gr-MDSC, reaching the level detected in the blood of healthy controls. In the group of patients after surgery (II group) and hormonal therapy (IV group) the results did not differ significantly (1.81 ±1.73 vs. 1.6 ±2.15) (Fig. 6), while in patients receiving combined therapy the level of Gr-MDSC was the lowest (0.69 ±0.70). In relation to Mo-MDSC, their level in the group of patients before treatment (I) and patients treated with hormonal therapy (IV group) was significantly higher than in the control group (15.40 ±10.94 in I group, 17.37 ±5.06 in IV group vs. 1.17 ±0.94 CTR; $p < 0.0001$ for both) (Fig. 6) and the highest level was observed in patients after surgery (15.40 ±10.94). Moreover, again, patients after combined therapy showed the lowest level of Mo-MDSC (5.50 ±5.40) (Fig. 6).

When comparing the PSA level with the treatment option the patients received, we found that the patients after surgical removal of the tumor or those receiving a combined therapy presented the lowest level of PSA (14.00±11.16 and 20.44±17.45, respectively), whereas the patients before any treatment had medium values of this marker (30.14±34.44). On the other hand, patients after hormonal therapy presented the highest PSA level (77.00 ±40.03; $p < 0.01$) (Fig. 6).

Discussion

Our study documents increased level of Gr-MDSC and Mo-MDSC in the prostate cancer patients'

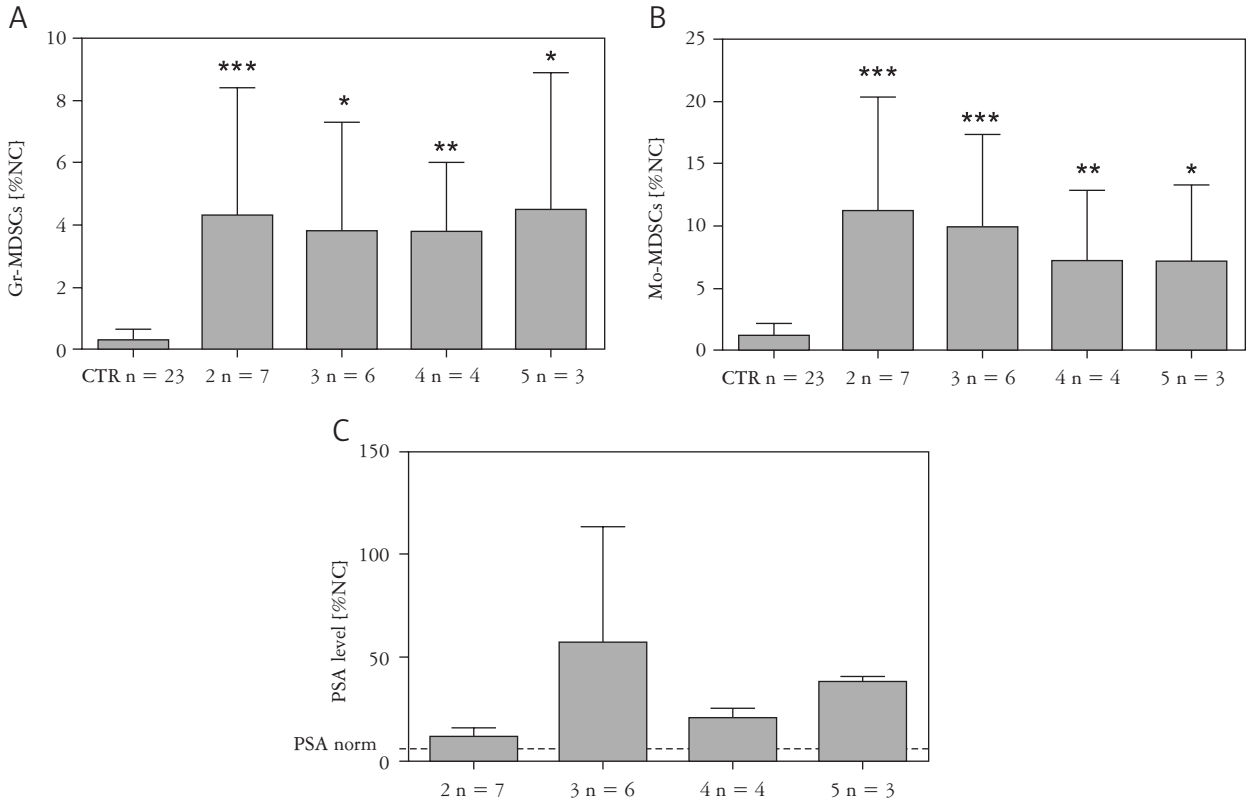

Fig. 4. Gr-MDSC (A) Mo-MDSC (B) PSA level (C) in healthy control subjects (CTR) and prostate cancer patients divided into four groups according to Gleason score. PSA normal level is indicated

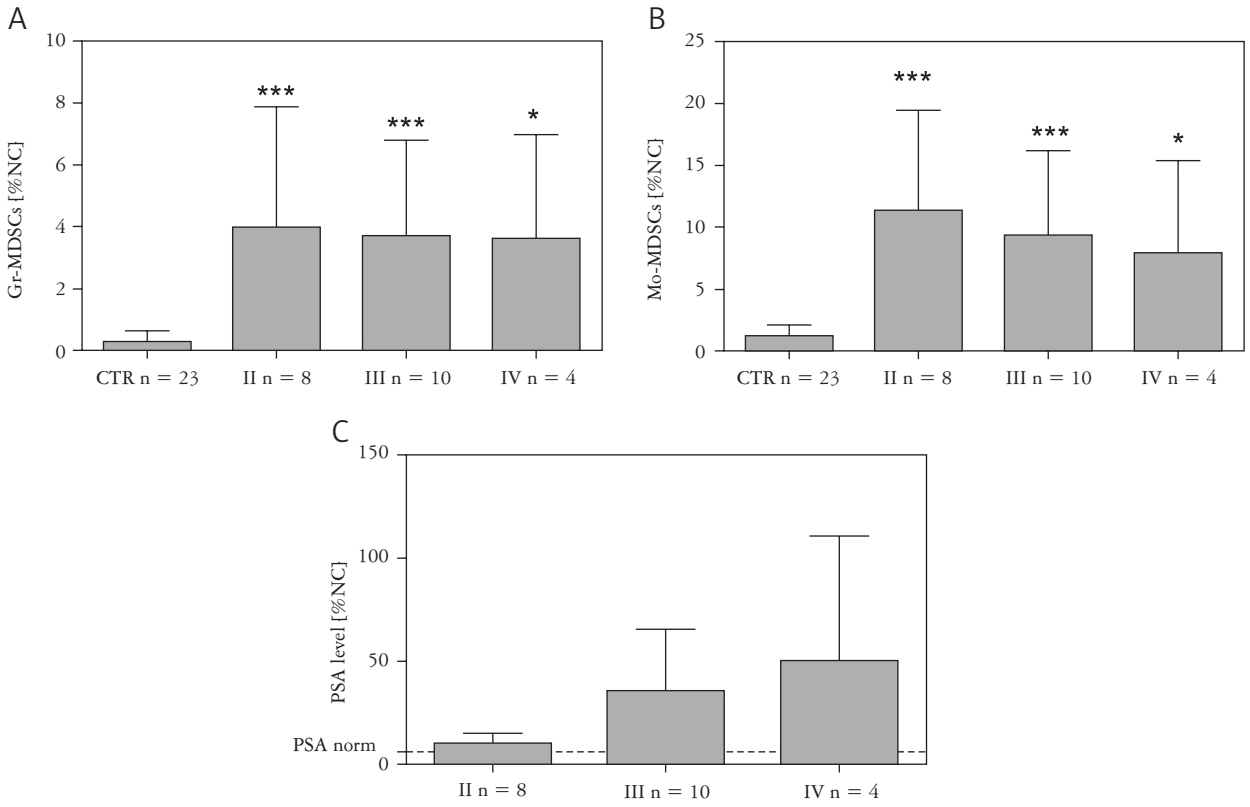

Fig. 5. Gr-MDSC (A) Mo-MDSC (B) PSA level (C) in healthy control subjects and prostate cancer patients divided into three groups according to the AJCC guidelines. PSA normal level is indicated

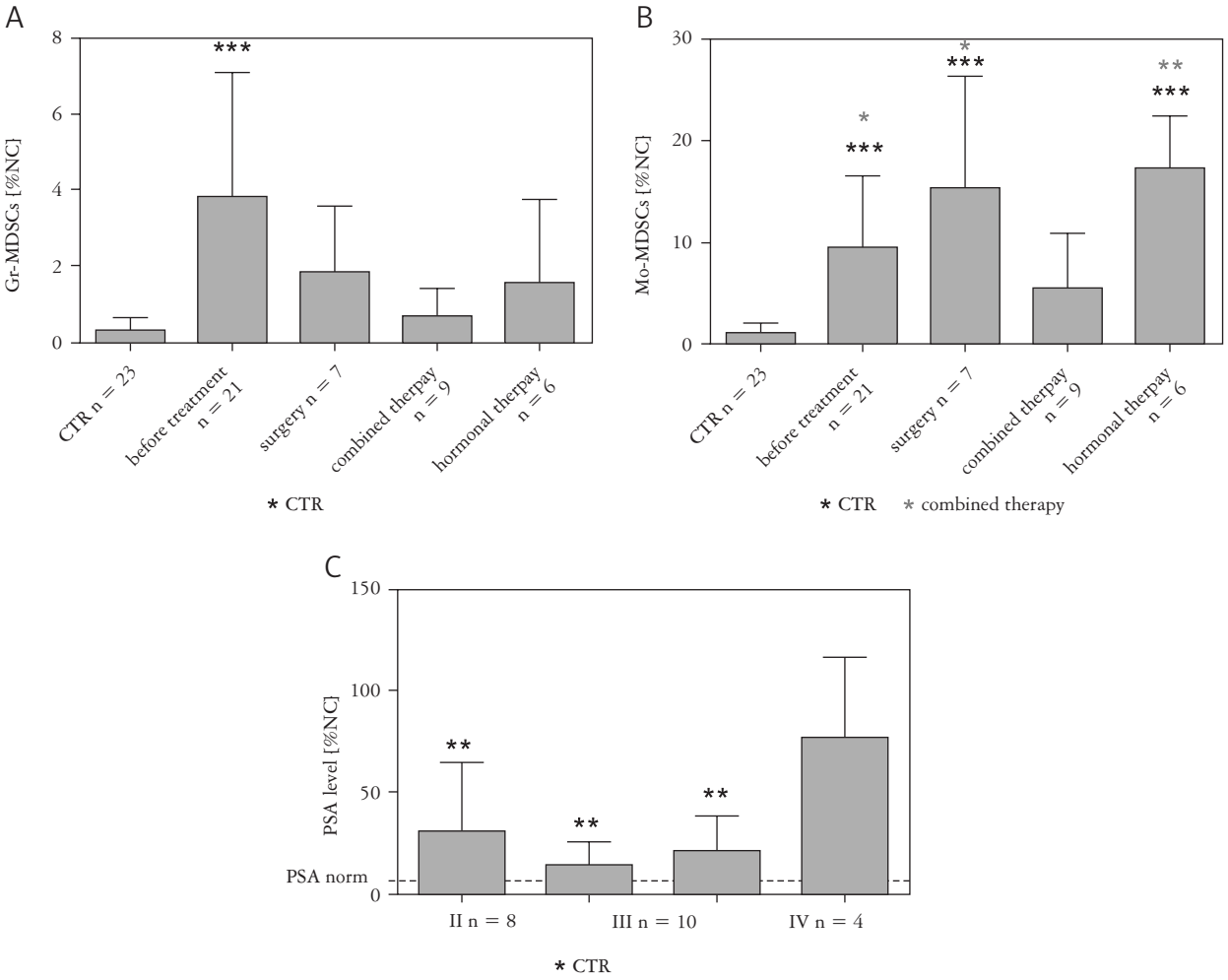

Fig. 6. Gr-MDSCs (A) Mo-MDSCs (B) PSA level (C) in healthy control subjects and prostate cancer patients divided in four groups depending on the treatment option. PSA normal level is indicated

blood, corroborating the previous reports [22, 23]. However, to the best of our knowledge the assessment of the level of MDSC populations in the blood of prostate cancer patients in respect to different forms of their treatment has not been performed so far. In our report we did not show any difference in the eMDSC level between patients and healthy individuals but data on this specific MDSC subset in prostate cancer is still missing in the literature. Although, in many types of cancer, Mo-MDSC mainly settle the tumor whereas Gr-MDSC prevail in the blood [24], in case of prostate cancer this relation is quite opposite, as higher level of Mo-MDSC than Gr-MDSC has been detected in peripheral blood [25]. This observation has been confirmed in our study. One of the first studies on MDSC in prostate cancer indicated that the level of Mo-MDSC in the blood of patients was positively correlated with the PSA level [26]. Our results corroborate this observation, however it is worth noting that the PSA level did correlate neither with the tumor grading nor with the clinical stage of the patients. It was already shown that the level of Mo-MDSC in peripheral blood of patients with prostate cancer increases with the progression of the tumor [27]. Knowing the role of MDSC in cancer development and progression one would expect that the treatment will reduce the level of these cells [22, 23]. Our results suggest that this is a case only for Gr-MDSC. Despite the treatment option the patients received, we observed significantly lower levels of Gr-MDSC in comparison to untreated group. This however, was not true for Mo-MDSC as their level in the blood of patients post-surgical treatment or after hormonal therapy did not differ significantly from the non-treated group. These results may suggest that surgery or hormonal therapy alone have no effect on the blood level of Mo-MDSC or these forms of treatment can even induce appearance of Mo-MDSC, as it was shown already for radiotherapy [28]. The study by Koga *et al*., demonstrated that prostate cancer patients with increased level of Mo-MDSC after the treatment had overall

worse survival [29]. Similar pattern, documenting an increased level of Mo-MDSC after therapy we observed also when analyzed blood from patients with colorectal cancer receiving surgery (manuscript in preparation). In case of prostate cancer, only patients receiving a combined treatment showed the level of Mo-MDSC comparable to this observed in a group of healthy donors and significantly lower than in a group before any treatment or receiving surgical or hormonal therapy alone. Although the others indicated a correlation between the level of MDSC and the clinical stage of prostate cancer patients [30], in our studies we did not observe such a connection. The level of Gr-MDSC or even Mo-MDSC was independent of the tumor grading and clinical stage, most likely due to the relatively small study groups. Similar pattern we observed in respect to the PSA level. In this case all patients presented the PSA level higher than the normal range, however no correlation between this parameter and the clinical stage or tumor grading was detected.

Up to now there have been no studies on the level of MDSC populations in the blood of prostate cancer patients in respect to different forms of treatment. Although, our work has a preliminary character and the groups of patients were small, it suggests that single method of treatment is not sufficient for the elimination of circulating MDSC, especially of monocyte origin. If this, however should be replaced by a combined therapy, further prolonged observations, including patients' survival analysis is required.

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The authors declare no conflict of interest.

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