Evaluation of the memory CD4+ and CD8+ T cells homeostasis during chronic venous disease of lower limbs

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Abstract: More and more is known about the role of venous wall abnormalities and valvular incompetence in the development of chronic venous disorders (CVD). Unfortunately detailed mechanisms of CVD pathophysiology are not well understood. Recent studies focus on involvement of the inflammatory process in the structural remodeling of venous valvues and venous wall. The aim of this study is to investigate and to document the memory T cells homeostasis in CVD patients. In this study we present lymphocytic changes in blood from varicose veins in terms of total CD4+ and CD8+ T cells and their particular subsets of memory T cells: T_N, T_{CM} and T_{EM}. Results suggest that immunological memory may be involved in the CVD development.

Key words: memory T cells, chronic venous disease, inflammatory process

Introduction

All cells if the immune system arise from multipotent stem cells which undergo differentiation, maturation and migration from primary lymphoid organs into secondary lymphoid organs [1,2].

CD4+ and CD8+ T cells subpopulations play important biological functions [3]. CD4+ cells are called helper T cells [4]. They generate or stimulate production of specific cytokines, and then they influence on inflammatory process [5,6]. CD8+ T cells are called cytotoxic T cells [7]. They have the ability for activation, suppression and cytotoxicity [8]. Heterogenous population of these cells is studied to characterize CD4+ memory T cells and CD8+ memory T cells with their subsets: central memory cells, effector memory cells and naive memory cells [9].

Memory T cells population have ability to survive in host after the completion of the immune response and they provide long-term protection against reinfec-

tion [10]. Speed and effectiveness of the secondary response upon reinfection depends on qualitative and quantitative differences of memory T cells in comparison with naive cells [11,12]. Precise mechanisms of memory T cells development steal are not well understood [13,14]. There are four potential mechanisms which may lead to the generation of memory cells [15]. The first model establishes the presence of separate naive precursors of effectors and memory T cells, in contrast to the second model which establishes that T cells constitute uniform population of precursors [16]. According to the third one, memory cells are derived directly from effector cells [17]. The influence of a level and time of stimulus duration for the generation of memory T cells considered in the fourth model of memory T cells formation [18].

Memory T cells are heterogenous in terms of their phenotype, anatomical distribution and functional properties [19]. The difference in occurrence of the CC chemokine receptor 7 (CCR7) and L-selectin (CD62-L) on the memory T cells surface is characteristic feature of these cells used to subdivide them into two functionally distinct T cells subsets [20,21]. Memory T cells may be defined as central memory T cells (CM)

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and effector memory T cells (EM). CM cells exhibits presence of the CCR7 and high expression of CD62L [22]. However on the EM cells lack is CCR7 and the expression level of CD62L is low [23]. Besides, identification of the memory T cells based on the expression of CD45RA on their surfaces, allows to differentiation of TEMRA among CD8+ T cells [24]. Other memory T cells subsets do not exhibit CD45RA expression on their surface. In the line with distinct expression of markers on memory T cells surface, they exhibit different localization [25]. CM cells mainly are present in secondary lymphoid organs and EM T cells in peripheral nonlymphoid tissues [26]. Differences in the phenotype of memory CD4+ and CD8+ T cells influence on different functions which these cells play in immune response. EM T cells provide a first line of defense against antigen. These cells are able to extravasate into peripheral tissues and they have a high potential of immediate effector functions. CM T cells are responsible for maintaining long-term protection upon reinfection. They have a high proliferative capacity and little potential of immediate cytotoxic function [27,28].

The pathophysiology of chronic venous disease (CVD) is not well understood, but it is known that always inflammatory changes are present. Moreover, the inflammatory process is investigated as the major cause of CVD [29].

Chronic venous disease (CVD) is a very common disease, which is significant social, medical and economic problem. The reason of the majority of CVD symptoms, (teleangiectases, varicose veins, edema, trophic changes, chronic leg ulceration), is prolonged venous hypertension caused by venous wall abnormalities and venous valves incompetence [30]. Recent advantages in understanding of CVD are focused on role of the leukocytes in the CVD pathogenesis [31]. In CVD patients, intense infiltration of leukocytes mostly lymphocytes and neutrophiles, was observed. Lymphocytic changes may suggest implication of immunological memory in the CVD pathogenesis. Chronic elevation of the venous pressure may cause leukocytes and endothelial cells activation [32]. The extravasation process of leukocytes is divided into rolling, activation, adhesion and transendothelial migration [33]. The extravation process of leukocytes is complicated and involves interactions between endothelial cells and leukocytes. During rolling leukocytes attach loosely to endothelial cells by L-selectin and E-selectin [34]. Leukocytes during the rolling process may meet various chemoattractants, (interleukin 8 (IL-8), complement split products, macrophage inflammatory protein (MP-1β) and N-formyl peptides), which biding with leukocytes surface receptors cause their activation [35]. The shedding of adhesion molecules from leukocytes surface

occur as the result of the activating signal. Level of soluble L-selectin was higher in CVD patients [36]. The activating signal induces also a conformational changes in the integrin molecules which results in increment of their affinity for Ig-superfamily adhesion molecules. The interaction of integrins and Ig-superfamily adhesion molecules causes firm adhesion of leukocytes to the endothelial cells and their migration through the endothelium [37]. Activated leukocytes migrate into venous wall and venous valve [38]. Leukocytes during the inflammatory process generate numerous substances (e.g.: proteolytic enzymes, free radicals and cytokines), which are responsible for the changes of the venous wall and venous valve, which lead to venous wall destruction and valvular incompetence [39].

The aim of the study was to investigate and to document the memory T cells homeostasis (central, effector and naive memory T cells) in CVD patients versus hospitalized abdominal hernia patients. We also evaluated their correlations.

Materials and methods

Patient samples. Group A: 9 patients, 7 women and 2 men aged 32-65, were included in the study. All patients were hospitalized for surgical removal, by crio-stripping, of varicose veins of the lower extremities due to chronic venous disorder (CVD). CVD severity was evaluated using the CEAP (clinical, etiologic, anatomic, pathophysiologic) classification. Two 10-ml EDTA blood samples were obtained from all patients prior to the surgery.

The first blood sample was obtained from a superficial forearm vein unaffected by CVD. The second blood sample was taken from a superficial lower limb vessel affected by CVD. Prior to obtaining blood from a lower limb venous vessel, each patient remained in an upright position for 20 minutes to provoke a venous stasis in the pathological superficial venous vessels.

Group B. 4 patients, 2 women and 2 men aged 19-35, hospitalized due to abdominal-wall hernia and scheduled for celotomy, were qualified for the study. None of the control group members displayed CVD signs. Blood samples were obtained from a superficial forearm vein unaffected by CVD directly before surgery.

Isolation of lymphocytes. Mononuclear cells were isolated from peripheral blood (PBMC). They were isolated by mechanical Ficoll gradient centrifugation.

Flow cytometry analysis. Fresh mononuclear cells were directly stained with anti-human CD4 APC Ab (APC labeled, clone Mouse $IgG_1\kappa$, BD Pharmingen), anti-human CD8β PE Ab (PE labeled, clone 2ST8.5H7, Beckman), anti-human CD28 FITC Ab (FITC labeled, clone CD28.2, BD Pharmingen), anti-human CD95 Ab (PE-Cy5 labeled, clone DX2, BD Pharmingen).

Briefly, 1×10^6 lymphocytes isolated by Ficoll were incubated with previously described Abs for 30 minutes at room temperature. After washing the cells twice in Dulbecco's phosphate buffered saline supplemented with 2% FCS and fixation in 1% paraformaldehyde, samples were analyzed by flow cytometry using The BD FACSCalibur™ system.

For the analysis the memory T cell population was identified on forward and side scatter for the lymphocyte population and on CD4 and CD8 expression. T_{CM} were identified as CD28+/CD95+,

T_{EM} as CD28-/CD95+ and T_N as CD28+/CD95-. The date are presented as percentages of total CD4 and CD8 or as percentages of T_N, T_{CM} and T_{EM} memory cells.

Statistical analysis. The normality of data was evaluated by the Kolgomorow-Smirnow test and non-normal distribution was observed in analyzed groups. Statistical analysis was performed using of the nonparametric U-Mann-Whitney test. Correlations were assessed using the Spearman correlation method. Values of $p < 0.05$ were considered as statistically significant. In calculations Statistica 8.0 software was used.

Results

After gating on lymphocyte region lymphocytes were identified as CD4+ T cells and CD8+ T cells and measured. In the next step T_N, T_{CM} and T_{EM} were characterized by the expression of CD28 and CD95 and were measured.

The quantitative evaluation of total CD4+ and CD8+ T cells and their particular subsets of memory T cells: T_N, T_{CM} and T_{EM} in peripheral blood in group A shows that the level of total CD4+ was higher than total CD8+ in peripheral blood. Among memory T cells subsets, the level of T_{CM} was the highest and the level of T_{EM} was the lowest (Fig. 1a). The level of total CD4+ and CD8+ T cells and their particular subsets of memory T cells: T_N, T_{EM} and T_{CM} was also measured in blood from varicose veins in group A. The level of total CD4+ was higher than total CD8+. High levels of T_{CM} were observed. The level of T_N CD8+ was higher than T_{EM} CD8+, whereas the level of T_N CD4+ was lower than T_{EM} CD4+ (Fig. 1b). Measurements of total CD4+ and CD8+ T cells and their particular subsets of memory T cells: T_N, T_{CM} and T_{EM} in peripheral blood in group B also show the higher level of total CD4+ than total CD8+ and that among memory T cells subsets the level of T_{CM} was the highest and of T_{EM} was the lowest (Fig. 1c).

In this study we also show the comparison between of total CD4+ and CD8+ and particular subsets of memory T cells: T_N, T_{CM} and T_{EM} in peripheral blood, among group A and group B. No statistically significant differences were found between group A and group B in terms of total CD4+ and CD8+ T cells and particular subsets of memory T cells: T_N, T_{CM} and T_{EM} (Fig. 2).

Moreover, we demonstrate correlations in group A between peripheral blood and blood from varicose veins in terms of total CD4+ and CD8+ T cells and particular subsets of memory T cells: T_N, T_{CM} and T_{EM} (Fig. 3). There were almost full correlations of total CD4+ T cells (r=0.91; p<0.05), total CD8+ T cells (r=0.96; p<0.05) and T_{CM} CD8+ cells (r=0.95; p<0.05). Furthermore, there were very high correlations of T_{CM} CD4+ cells (r=0.76; p<0.05), T_{EM} CD4+ cells (r=0.83; p<0.05) and T_{EM} CD8+ cells (r=0.71; p<0.05). Whereas in T_N CD4+ cells (r=0.68; p<0.05)
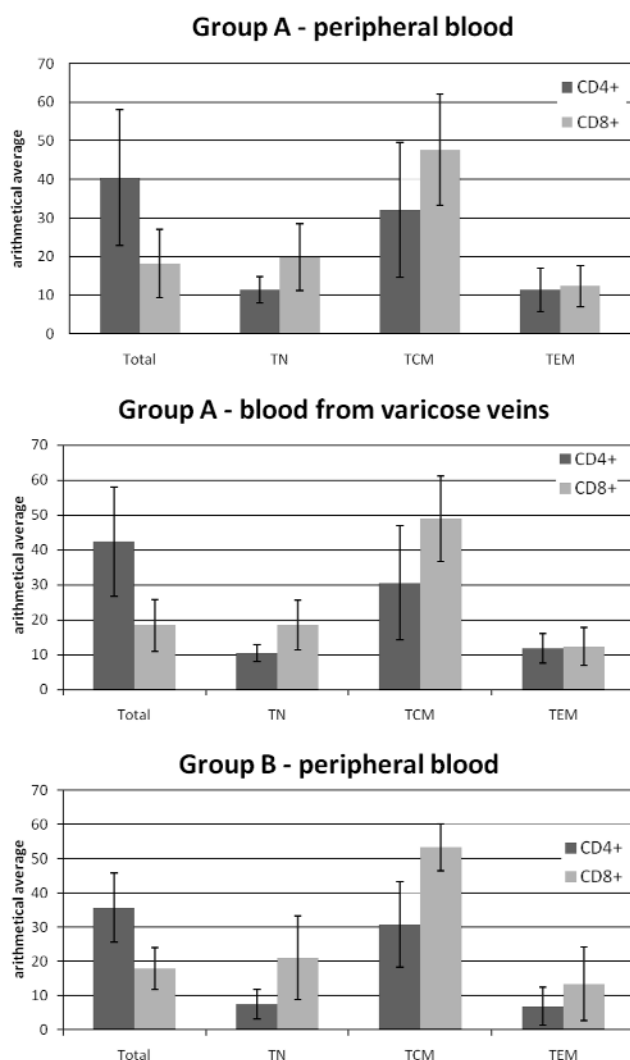

Fig. 1. The quantitative evaluation of total CD4+ and CD8+ and particular subsets of memory T cells: T_N, T_{CM} and T_{EM} in group A and group B.

and T_N CD8+ cells (r=0.63; p<0.05) were high correlations. All correlations were statistically significant besides correlation of T_N CD8+ cells.

Discussion

Chronic venous disease is very significant problem among Western population [40,41]. Chronic venous disease is one of the most common disease, but precise mechanisms of its pathophysiology remains not well known [42]. The role of the valvular incompetence and venous wall abnormalities in the pathophysiology is well understood. Less is known about the reasons which lead to the development of these changes [43].

Recent studies indicate that always there is present an inflammatory process during the development of CVD [44]. In this study using identification of total CD4+ and CD8+ and particular subsets of memory T
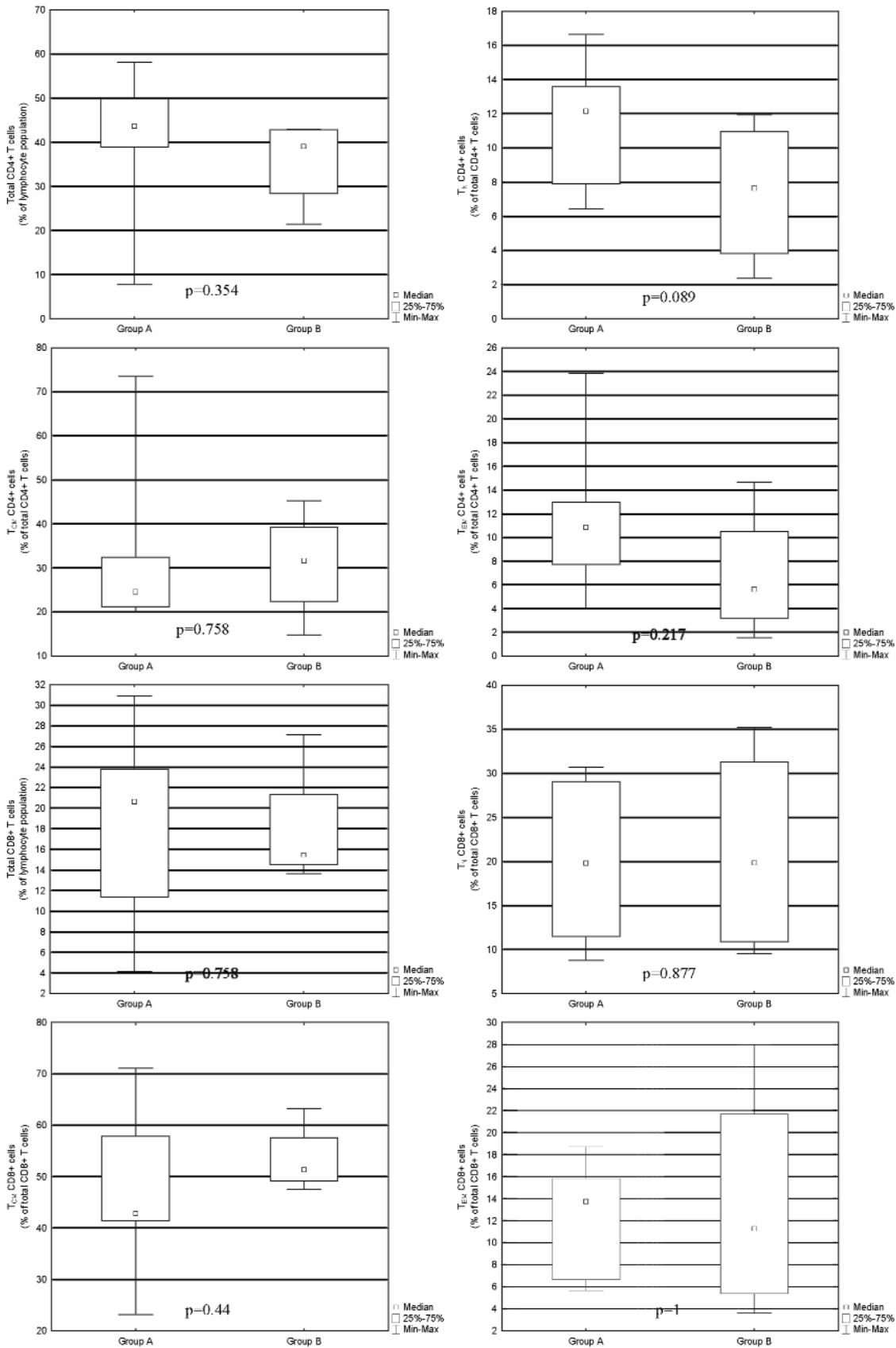
Fig. 2. The comparison between total CD4+ and CD8+ and particular subsets of memory T cells: T_N, T_{CM} and T_{EM} in peripheral blood, among group A and group B.
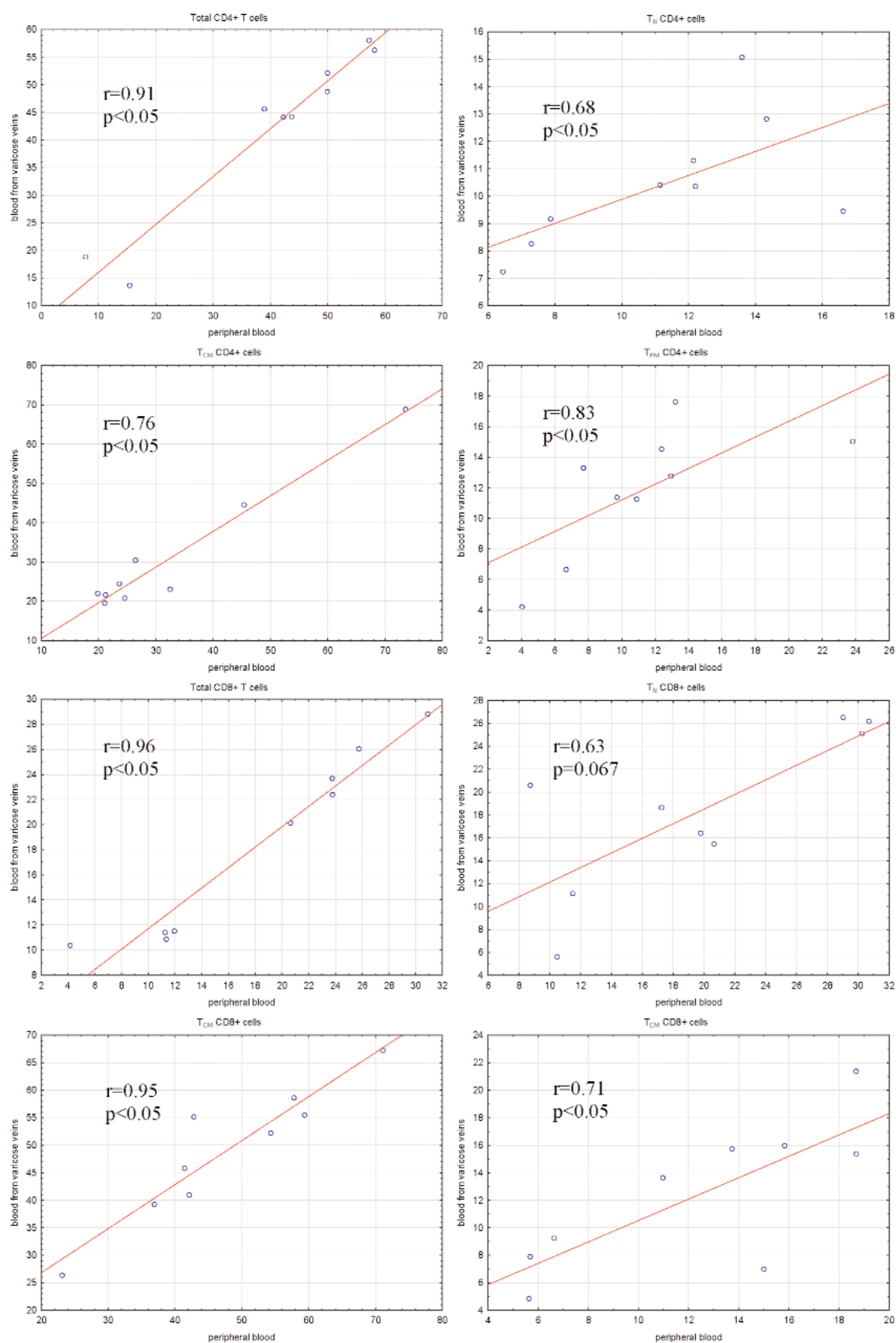
Fig. 3. Correlations in group A between peripheral blood and blood from varicose veins in terms of total CD4+ and CD8+ and particular subsets of memory T cells: T_N, T_{EM} and T_{CM}.

cells: T_N, T_{CM} and T_{EM}, based on the expression of CD4, CD8, CD28 and CD95, we show the memory T cells homeostasis in CVD patients versus hospitalized abdominal hernia patients.

We have demonstrate that no statistically significant differences were between group A and group B in terms of total CD4+ and CD8+ T cells and particular subsets of memory T cells: T_N, T_{CM} and T_{EM} in peripheral blood. While, there were statistically significant difference between peripheral blood and blood obtained from varicose veins in terms of previously described T cells subsets. These results are important implication about the presence of local inflammatory conditions.

Results presented in this study show enhanced values of T cells subpopulations in blood from varicose veins which can be regarded as lymphocytic changes. Measurements of particular subsets of memory T cells: T_N, T_{CM} and T_{EM} in blood from varicose veins show that the level of T_{CM} the highest and the level of T_{EM} was the lowest. These findings lead us to speculate that immunological memory T cells may be involved in the CVD pathogenesis.

Moreover, the result of the increased level of lymphocytes in blood obtained from varicose veins is related to studies focused on intense inflammatory cells infiltration (mainly lymphocytes) in the venous valves and venous wall [45]. Leukocytes undergo activation during chronic elevation of the blood pressure in veins [46]. Activated cells migrate into valvular and venous wall and may be responsible for the changes of the venous wall and venous valve. Several studies show enhanced infiltration of lymphocytes, monocytes, macrophages and neutrophils into venous wall [32]. Inflammatory cells may release proteolityc enzymes, free radicals and cytokines responsible for the destruction of tissue [47,48].

Particular understanding of the inflammatory process in the development of chronic venous disease may be significant for treatment of that disease or even for its prevention.

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