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B-Lymphocytes level in Peripheral Blood among Patients with Metabolic Syndrome and Diabetes Mellitus Type 2

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Abstract

A considerable suggestion exists between the leukocyte count, lymphocyte (lymph) dysfunction and the possibility of developing DMt2. Aim of the study was to investigate the CD19 +expression of B- lymph in peripheral blood in patients with metabolic syndrome (MS) without and with Diabetes mellitus type 2 (DMt2). A prospective, one-year, comparative, observational study was performed. In 26 MS patients without DMt2 (n_1=26) and 69 with DMt2 (n_2=69) were measured and compared the levels of leucocytes and CD19+ B- lymph subtypes in peripheral blood by flow-cytometric analysis. The leucocytes count is in normal range in both groups with MS. DMt2 patients have a higher leucocytes count (n_1=6.93±1.92.10^9 vs n_2=7.27±1.85.10^9; p= 0,04) in the referent range. The percentage of the

common lymphocytes population was also higher among n_1 patients compared to n_2, but without significant difference in their absolute count [(n_1=38,26%±7,43% vs. n_2=33,42%±7,08%) and (n_1=2.58±0.8.10^9 vs. n_2=3,45±0,67.10^9)]. The percentage ratio and absolute count of CD19+ B- lymphocytes subtypes in patients from both groups was lower than referent values, but without significant difference between the two groups.The levels of CD19+B-lymphocytes in peripheral blood are lower in patients with MS irrespective of the level of glycaemia.

Key words: Metabolic syndrome, B- lymphocytes, type 2 diabetes mellitus risk, flow – cytometry.

Introduction

During the last 30 years the stereotypical concept for DMt2 has changed: from a classic metabolic to a multifactor chronic inflammatory disease. Abdominal obesity has not been only a major factor for the progress of insulin resistance (IR), but also responsible for the activation and persistence of low-grade chronic inflammation among patients with MS and DMt2 [1]. Adipose tissue macrophages have kept the persistency of the chronic inflammatory response. The ongoing inflammatory state has been a result of disturbance in the balance between anti-inflammatory and pro-inflammatory cytokines which are under a genetic control.

Some uncertainity exists concerning the causative role of chronic subclinical inflammation in the pathogenesis of the DMt2. One supposed mechanism involved in IR could be the ability of inflammation *per se* to interrupt the insulin signaling in the liver by pro- inflammatory molecules. The other suggestion has been directed to the manifestation of B-cells dysfunction by the possibility of inflammation to promote the pancreatic beta-cells death. It is thought that pro-inflammatory cytokines such as interleukin (IL) -6 and tumor necrosis factor-α (TNF-α) activate immunologic reactions, which may shift to specific autoimmune phenomena [2].

The role of anti-inflammatory molecules has also been an area of specific interest. One of them, IL-10 is an important anti-inflammatory cytokine involved in the regulation of innate

immune response. It has been a potent blocker on the macrophages- and lymphocytes-mediated inflammation and the production of pro-inflammatory cytokines [3]. The Leiden 85-Plus Study has shown that low pro- inflammatory response is associated with MS and DMt2, the odds ratio for type 2 diabetes is 2.7 if subjects have lowest IL-10 production [3].

White blood cells (WBC) are a non specific inflammatory marker. A considerable suggestion exists about the relationship between the WBC count and the risk of developing of DMt2. The results from epidemiological studies establish an association between WBC count and diabetes risk [4]. The results of meta-analysis from 20 cross-sectional and prospective cohort observational studies have shown that WBC count is positively associated with the risk of T2D. Its rise in individuals with abdominal obesity is associated with 1.5-fold increase risk of DMt2 [5]. The association of each of the subfractions of WBC has also been studied in concern to diabetic susceptibility. Total granulocyte and lymphocyte without monocyte counts are also proved to be significantly associated with T2D [6].

The role of adipose tissue monocytes and macrophage in pathogenesis of IR and DMt2 development has been elucidated mostly by “in vitro” researches [7]**.** Relatively few works are directed to study B- and T –lymphocytes cytokine production and their possible role to promote adipose tissue inflammation in humans. A study performed in obese mice has shown overproduction of pro-inflammatory molecules and has supposed that B cells might be the critical regulators of T-cells function to promote the pro-inflammatory cytokines production. They demonstrated that human B-cells have increased DMt2- associated T-cell inflammatory response by contact-dependent mechanism. According to the similarity in the results from obese IR mice and human immune cells, the authors summarize that B cells increase inflammation in obesity through two pathways: 1) by regulation of an inflammatory T-cells function and 2) with production of pro-inflammatory cytokines [8].

Polygene models have shown that knockout mice with deletion of the gene for the heavy immunoglobin’s chain and artificial B-cells deficit can’t develop DMt2 [9]. This has lead to the suggestion that patients with DMt2 have B- lymphocyte cell deficiency [9]. It also has been established that CD+ regulatory B lymphocytes predominantly produce the anti-

inflammatory cytokine IL-10. Decreased production of IL-10 after stimulation of pattern-recognition receptors (PRRs) on the immune cells recognizing pathogen-associated molecular patterns (PAMPs) has been noticed in diabetic patients compared to healthy subjects [9]. Low production of IL-10 in response to lipopolysacharides has been associated with increased risk for developing DMt2 in adult patients [10].

The aim of the study was to investigate the CD19 +expression of B- lymph in peripheral blood in patients to with MS and DMt2 .

Patients and methods

A prospective, one-year, comparative, observational study was performed among 95 patients with MS. All patients were involved in the study according to specific inclusion and exclusion criteria. Patients with anamnesis and clinical data for acute or exacerbated chronic inflammatory process and autoimmune diseases, with acute metabolic diabetic complications (ketoacidosis, hyperglycemic hyperosmolar conditions, hypoglycemia) and/or taking corticosteroid or immune- suppressive and immune-modulating treatment had been excluded. Patients of both genders were divided into two groups according to the glycemic status after performing of 75-gr. OGTT: first group = 26 patients with MS without DMt2 (n_1=26) and second group = 69 with DMt2 (n_2=69).

The diagnosis of MS was evaluated by the IDF criteria from 2010 [11]. The DMt2 was established according to the WHO diagnostic criteria, from 2011 [12]. The study was approved by the local medical ethic committee. Informed consent was obtained from all participants.

Anthropometric data was taken including height (meters) and body weight (kilograms) for determination of body mass index (BMI). The waist circumference (cm) was measured on a horizontal plain located in the middle between the lower edge of the 12th rib and the upper edge of the iliac bone, with accuracy up to 0.5 cm.

The arterial blood pressure (mmHg) was examinated in a sitting position under

standard conditions, after a 5-minute rest, at an interval of 5 minutes between two successive measurements.

Blood samples for WBC, blood glucose, total cholesterol, HDL- cholesterol and triglycerides were obtained in a fasting state at 8 am. Total cholesterol, HDL-cholesterol and triglycerides were assessed by the enzyme colorimetric method (GPO- PAP; Biocon® Diagnostik), LDL - cholesterol was calculated by the Friedwald formula (LDL-cholesterol = general cholesterol - HDL-cholesterol - triglycerides/2.2).

Automated blood cell count for leukocytes including lymphocytes, monocytes and granulocytes was performed on the Micros 60 (HORIBA ABX Diagnostics, France) after lysis of erythrocytes.

Fresh venous blood was drawn into sodium-heparin tubes and the results were obtained within 2 hours. Leukocytes were analyzed by flowcytometry using a dual-laser FACS Calibur cytometer (Becton Dickinson, Heidelberg, Germany) and Cellquest software (Becton Dickinson). Briefly, blood cells were stained with fluorescence-conjugated antibodies in two different colors. After lysis of erythrocytes (Lysis buffer; Becton Dickinson) and two washes, stained PBMC were re-suspended and fixed with CellFIX (BD Biosciences). Ten thousands of lymphocytes were collected in a forward scatter/side scatter (FSC/SSC) lymphocyte gate and saved together with the monocytes and granulocytes. The flowcytometer was calibrated daily with appropriate single-stained samples for setting compensation and acquired data was analyzed by FACSComp software©2007 Becton Dickinson. Fluorescence-conjugated antibodies were used to identify cell populations CD19 (B cells). CD19 is expressed throughout B-cell development until terminal plasma cell differentiation.

Statistical analyzes

All analyses were performed using STATGRAPHICS Centurion XV.I. Data were presented as their mean value and their standards deviations (means ± SD) or as individual data and median values. Comparisons between groups were done using: Independent sample t-test for parametric comparison of the two means, Kolmogorov Smirnov for a non-parametric

comparison and Mann-Withey tests for the test median of two groups. Two-sided P values ≤0.05 were considered to indicate statistical significant differences. The Pearson (r) correlation for measurement the strengths of association between two variables were also done.

Results

The basal clinical characteristics of the patients is shown on Table 1. All participants in the study met clinical (BMI, blood pressure, waist), biochemical (cholesterol, LDL-cholesterol, triglycerides) and IDF criteria for MS. There are statistically significant differences between the two groups according to the age, waist circumference, fasting blood glucose and triglyceride levels. Patients with DMt2 are older than those with MS (n_2 = 56.58±9.36 vs n_1= 40.15±13.80; P<0.05) and have bigger waist circumference (n_2= 56.58±9.36 vs n_1= 40.15±13.80; P<0.05) higher fasting glucose levels (n_1= 5,58 ± 2,23 vs n_2=8,96± 3,91 ;P<0.05) and triglyceride levels (n2=2.50±1.38 mmol/l vs n1=1.44±0.79 mmol/l; P<0.05).

Whole leucocyte count among the patients of the two groups is in normal range. A statistically significant difference is found in the mean values of the WBC count between the two groups. Patients with DMt2 have higher leucocytes count, also inside of the referent range than normoglycemic patients without DMt2 (n_1=6.93±1.92.10^9 vs n_2=7.27±1.85. 10^9; P= 0,04). We establish statisticaly significant differences in the distribution of the total lymphocytes populations (B- lymphocytes and T- lymphocytes) counting as a per cent and an absolute count among the two groups. [(n_1=38.26±7.43% vs n_2=33.42±7.08.%; P< 0.05) and respectively (n_1=2.58±0.8.10^9 vs n_2=3,45±0,67.10^9; P<0,05)]. (table.2).

The results of flow-cytometric analysis show lower levels of CD19+ lymphocytes subpopulations than the normal ranges in both groups. The B-lymphocyte subpopulation (CD19+) in patients with DMt2 is lower compared with the same cells subtype in patient with MS without DMt2. Moreover, the differences between them have been statistically significant (tabel.3).

Tabl.1 Basal clinical characteristics of patients

Parameters	**Non- DMt2 MS patients** (n1=26)	**DMt2 MS patients** (n2=69)	**Significance P<0.05**
Age(years)	40.15±13.80	56.58±9.36	P*
BMI (kg/m^2)	34.96 ±7.76	34.24±6.08	
Waist (sm.)	107.39±17.12	112.76±14.82	P*
Systolic Blood pressure (mmHg)	127.12±14.08	133.82±16.26	
Diastolic Blood pressure (mmHg)	82.5±10.8	82.87±8.61	
Fasting glucose (mmol/l)	5,58 ± 2,23	8,96± 3,91	P*
Total cholesterol(mmol/l)	5.4±0.97	5.59±1.24	
HDL-cholesterol(mmol/l)	1.38±0.29	1.20±0.36	
LDL cholesterol(mmol/l)	3.40±1.05	3.21±1.17	
Triglycerides(mmol/L)	1.44±0.79	2.50±1.38	P*

p* <0.05

Tabl.2 Whole and differentiated white blood cells

Parameters	**Non- DMt2 MS patients** (n1=26)	**DMt2 MS patients** (n2=69)	**References range**
White blood cells ($10.^9$)	6.93±1.92	7.27±1.85	3.5-10.0
Lymphocytes (%)	38.26±7.43	33.42±7.08	20.0-48.0
Lymphocytes-absolute count ($10.^9$)	2.58±0.8	3.45±0.67	1.2-3.2
Monocytes (%)	6.42±1.14	6.88±1.33	1.0-11.0
Monocytes-absolute count ($10.^9$)	0.37±0.11	0.45±0.15	0.3-0.8
Granulocytes(%)	55.32±7.87	59.7±7.42	40.0-70.0
Granulocytes-absolute count ($10.^9$)	3.84±1.23	4.5±1.4	1.2-6.8

There is no relationship between CD19+ expression and blood glucose, total cholesterol, HDL – cholesterol, LDL -cholesterol and triglycerides in the both groups. We have found negative correlations between absolute count of the CD19+, BMI and waist circumference in patients with MS without DMt2 (r= -0.49) and with DMt2 (r=-0.47).

Discussion

The results from the current research, carried out with MS patients show total

Tabl 3. CD19+ lymphocytes subpopulations

	Non- DMt2 MS patients (n1=26)	**DMt2 MS patients (n2=69)**	**References range**
B Lymphocytes CD19+ (%)	8.72±4.31	7.36±3.64*	11-16
B Lymphocytes CD19+ (absolute count)	223.08±130.3	186.02±101.78*	200-400

*indicates significant difference between DMt2 and low- normal levels, ($p<0.05$)

‡indicates significant difference between DMt2 and low-normal levels ($p<0.05$)

WBC count in reference range and normal WBC subfraction distribution irrespective of the presence of diabetes. The comparison in absolute WBC count confirms that the patients with Although the WBC is inside of the referent range in patients with DMt2 they have leucocytes count higher than normoglycemic patients without DMt2. The diabetic patients also have higher count of total lymphocytes populations and lower B-lymphocytes count. A negative associations has existed between BMI, waist circumference and CD19+ expression among the patients with MS independent of glycemic levels. One possible explanation of our finding could be associated with the degree of abdominal obesity. The patients from both groups have a similar BMI, but diabetic patients have bigger waist circumference. The visceral fat mass produces adipokines and other products of adipocytes, causing the recruitment of macrophages and other immune cells. (13). We could speculate that certain adipose abdominal tissue over-deposition causes metabolic dysfunction with impairment in normal metabolic function and deregulation of the immunity, proved with increased WBC count, lymphocytes populations and decreased CD19 +expression.

Our results of total WBC in normal range are in contrast from literature date. Over the

last 10 years, several epidemiologic studies have proved the link between leucocyte count and the appearance of MS among Asian population [14 - 16]. A retrospective cohort study [17] reports an increase in the frequency of MS with increasing of leucocyte count. There are also some prospective studies, proving leucocytes as a prognostic marker for the development of MS and DMt2 [18 – 20]. Some researchers have reported for association between obesity, DMt2 and higher leucocytes. They did not found a similar correlation in normoglycemic non-obese DMt2 individuals [6, 21].

Apart from the significance of WBC count as a classical diagnostic marker for acute inflamation and tissue destruction, its meaning has been discussed as a predictive parameter of endothelial disturbances in MS. The rise of WBC is closely associated with the progression of the atherosclerotic process and with the death-rate caused by cardio-vascular diseases [18]. We have difficulties to explain the certain role of the lower CD19 + expression on B lymphocytes in peripheral blood in MS. One possible suggestion is proposed by the lower production of anti-inflammatory molecules. Van Exel and co-workers have found a link between the reduced CD19+lymphocytes production of IL-10 and the higher risk of DMt2 in experimental models [10]. DeFuria has confirmed the promotive role of B lymphocytes for the development of type 2 diabetes in insulin resistant patients [22]. They also have found that the percent and absolute count of B-lymphocytes had been lower than the under limit reference range and have proved the negative correlation between levels of CD19+ lymphocytes and BMI.

In our study there are some limitations according to the lack of a control group of healthy individuals without MS and the relatively small number of studied populations.

Conclusions

The study has indicated B- lymphocyte dysfunction in patients with MS and DMt2, confirmed with the lower levels of B- lymphocytes in peripheral blood in patients with MS and DMt2. We have supported the promoter role of visceral fat in the process of developing and maintaining dysregulation of some metabolic and pro-inflammatory functions leading to

DMt2. A future prospective study with an appropriate design is necessary to be performed among a large cohort of metabolically healthy non-obese and metabolically non-healthy obese patients.

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