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# Flow cytometry analysis of PPARα receptors in metabolic syndrome

## Studiul receptorilor PPARα prin metoda citometriei în flux în sindromul metabolic

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## Abstract

***Introduction***. *Metabolic syndrome (MS) is a cluster of distinct metabolic alterations with an increased cardiovascular risk. Peroxisome Proliferator-Activated Receptor - Alpha (PPARα), member of the nuclear receptor superfamily of transcription factors, is critically involved in the management of lipid metabolism during homeostasis or inflammatory stresses in various cell types and represents one of the therapeutic targets in MS. We analysed the PPARα expression in leukocytes of pacients with MS, in order to address PPARα involvement in these group of diseases.* ***Material and method***. *Our study included 57 adult patients recruited under informed voluntary consent, investigated in order to establish whether they present MS, according to International Diabetes Federation (IDF) European guidelines and grouped in 2 lots: the MS Lot (26 patients) and control group, non-MS Lot (31 subjects). Common clinical and laboratory parameters targeted in MS evaluation were determined for all the studied cases. The expression levels of 2 molecules, PPARα and CD36 were evaluated in various circulating leukocyte populations of these patients by an optimized flow cytometry method. Statistic analysis clarifying the significance of value differences for various parameters measured was performed under SPSS and simple statistical tests (Pearson, t-Student, Chi -test).* ***Results and discussion***. *The fluorescence staining for PPARα were significantly dimmer when comparing the cellular expression in eosinophils ($p<0.05$) of MS versus the Control group of subjects.* ***Conclusions***: *Our study is the first to show that circulating eosinophils display significantly reduced PPARα protein expression in MS patients. The differences in key molecule expression in circulating leukocytes (like PPAR species, CD36, and other) might be evocatory for the endothelial dysfunction and obesity and might be of use in the therapeutic decision.*

***Keywords***: *PPARα; metabolic syndrome; leukocytes; eosinophyls*

## Rezumat

***Introducere***. *Sindromul metabolic (SM) reprezintă o asociere de alterări metabolice independente cu risc cardiovascular crescut. Receptorii activați de inductorii proliferării peroxizomilor-alpha (PPARα), membri ai*

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*superfamiliei receptorilor nucleari cu rol de factori de transcripție , sunt implicați în reglarea metabolismului lipidic și inflamație în diferite tipuri de celule și reprezintă una din țintele terapeutice în SM. Am analizat expresia PPARα în leucocitele pacienților cu SM, îndreptându-ne spre implicarea PPARα în această patologie.* ***Material și metoda****. Studiul s-a desfășurat pe un număr de 57 pacienți adulți recrutați după semnarea consimțământului informat, investigați pentru evidențierea SM conform criteriilor Federației Internaționale de Diabet (IDF) și care au fost grupați în două loturi: lotul SM (26 pacienți) și lotul de control, fără SM (31 subiecți). Au fost determinați parametrii clinici și de laborator utilizați curent în evaluarea SM. În populațiile de leucocite circulante ale pacienților au fost evaluate nivelurile de expresie a două molecule, PPARα și CD36, printr-o metodă optimizată de citometrie în flux . Semnificația diferențelor dintre valorile parametrilor măsurați a fost stabilită prin analiza statistică, utilizând programul SPSS și teste statistice simple (Pearson, t-Student, Chi-test).* ***Rezultate și discuții****. Când am comparat nivelul de expresie a PPARα în eozinofile, am obținut a reducere semnificativă a intensității medie de fluorescență ($p<0.05$) pentru PPARα în lotul MS versus lotul martor.* ***Concluzii****. Studiul nostru este primul care arată că nivelul de expresie a PPARα în eozinofile este diminuat semnificativ la pacienții cu SM. Diferențele de expresie ale acestor molecule cheie în leucocitele circulante (ca diferite specii de PPAR, CD36 și altele) ar putea deveni evocatoare pentru disfuncția endotelială și obezitate și ar putea fi utile în elaborarea unei decizii terapeutice.*

***Cuvinte cheie****: PPARα; sindrom metabolic; leucocite; eozinofile*

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## Introduction

A close link between metabolic alterations (diabetes mellitus, dyslipidemia, obesity) and cardio-vascular diseases has been consistently documented during the past few decades (1, 2). Various complex pathologies have been described since 1988 as aggregation of metabolic factors, wherefrom we mention: the Reaven syndrome, the metabolic X syndrome, the insulin resistance syndrome, the dysmetabolic syndrome, and the metabolic syndrome (MS) (3). MS is nowadays acknowledged as a complex pre-diabetes clinical condition derived from a continuous accumulation of independent metabolic alterations with an increased cardiovascular risk. Eventually, these changes lead to the initiation of the atherosclerotic and inflammatory degenerative process wherein the central pathogenic element is represented by the compensatory hyperinsulinemia and insulin resistance (4). MS affects more than 25% of the population, and about two-thirds of MS patients are liable for a major cardiovascular event (5, 6). Therefore, optimized protocols for MS prevention and therapy are urgently required. However, the therapeutic targets are frequently difficult to achieve, despite the more recent focus on the proper management of cardio-metabolic risk factors, as recommended by current medical guidelines (7).

One group of cellular proteins, **Peroxisome Proliferator-Activated Receptors-Alpha (PPARα)** has recently drawn a great deal of attention due to their significant role in the regulation of lipids and lipoproteins metabolism, chronic inflammation, and atherogenesis. Atherosclerosis, frequently defined as a consequence of progressive dependence between adiposity and insulin resistance (8), may be nowadays linked to the activation of PPARα receptors. These molecules are transcription factors involved in lipid metabolism, as their activation trigger a lower serum level of triglycerides (TG), an increased serum concentration of HDL-cholesterol (HDL-chol), a modulated proinflammatory status, and decreased insulin resistance, leading to an overall protective effect (9, 10).

It has been reported that the key contribution of PPARα in controlling atherogenesis relies on their involvement in lowering inflammation (11, 12, 13). Having all the above in mind, our aim was to establish a relationship between the cell-expression level of PPARα and various clinical markers of MS, using a rapid and accurate method (flow-cytometry) and targeting cells from accessible specimens (peripheral blood). The premise of the study was that novel, easily accessible assays for the early diagnosis of MS are required.

Another cell component that may serve as early landmark for MS diagnosis is CD36, a cell-surface glyco-protein expressed by monocytes (Mo), macrophages, endothelial and smooth muscle cells, acting as a „scavenger" receptor for oxidized LDL and free fatty acids. The role of CD36 in dyslipidemia, atherosclerosis, and diabetes mellitus was documented in studies showing that its increased gene expression correlates with elevated proinflammatory responses (14, 15).

The second aim of the current study was to evaluate the expression of PPARα in correlation to the expression of CD36 on circulating Mo and relative to the clinical and biochemical profiles of MS diagnosed human subjects, comparative with non-MS controls.

## Material and method

***The study group***

The study included 57 voluntary patients diagnosed at the Individual Medical Practice CMI Elena Popa Iasi and Hospital Ambulatory Care "Sf. Spiridon" Iasi. The study was approved by the Ethics Committee of the University of Medicine and Pharmacy "Grigore T. Popa" of Iasi, based on the informed consent of patients, according to World Medical Association, Helsinki Declaration (2013 revision, Brazil).

***MS components considered***

The study group was subject to biochemical and clinical analysis in order to identify the MS patients according to International Diabetes Federation (IDF) guidelines. **IDF diagnostic criteria of MS diagnosis** (5) **were: central obesity** - defined by waist circumference (WC) with ethnic variability or body mass index (BMI) > 30 kg/m$^2$ **plus any two of the following four criteria:** 1. Increased levels of serum triglycerides (TG) ≥ 150 mg/dl (1.7 mmol/ L) or specific treatment for hypertriglyceridemia; 2. Reduced high-density lipoprotein cholesterol- HDL-chol < 40 mg/dl (1.03 mmol/ L) for men and < 50 mg/dl (1.29 mmol/ L) for women or specific treatment for these dyslipidemia; 3. Blood pressure (BP) – systolic blood pressure (SBP) ≥ 130 mmHg or diastolic blood pressure (DBP) ≥ 85 mmHg or treatment for previously diagnosed hypertension; 4. Raised fasting plasma glucose ≥ 100 mg/dl (5.6 mmol/L) or previously diagnosed type 2 diabetes.

Within the study group there were two clusters of patients: Control group (non - MS) and MS Lot. The following actions were then performed sequentially: A. Cardiovascular disease and/or diabetes mellitus hereditary antecedents, physiological and personal past history, as well as the medication supplied were documented for each subject; B. Anthropometric data (body weight, height, WC, BMI), SBP, and DBP were recorded and assessed; C. Venous blood collection was performed for standard biochemical tests relevant for the glucidic metabolism (glycemia), lipid metabolism (total cholesterol, high-density lipoprotein cholesterol – HDL-chol, low-density lipoprotein cholesterol – LDL-chol, TG), renal function (urea, creatinine, uric acid) and hepatic function (transaminases, gamma-glutamyl transpeptidase).

Flow-cytometry-based evaluation of relative expression levels of PPARα and CD36

From the venous blood samples collected on EDTA, the PPARα nuclear receptor relative expression levels was determined within various types of circulating blood cells, using an "in house" optimized flow cytometry protocol.

In short, each blood sample was first left on the roller for 30 minutes, and then 50 µL aliquots were distributed in 1.5 mL eppendorf tubes. Red blood cells in each tube were lysed (FACS Lysing solution, Becton Dickinson, BD), then cells from each tube were washed twice with a neutral buffer (FACS Flow solution Becton Dickinson, BD), resuspended in 250 µL solution for surface membrane fixation and permeabilization (Cytofix Cytoperm solution, Becton Dickinson, BD), followed by an incubation step of $4^0$C for 20 minutes. After an additional washing step with 1 mL FACS-Flow solution supplemented with 1% Foetal Calf Serum - FCS (Sigma), the supernatant was carefully removed, leaving no more than 30 µL of cell suspension in each tube. 1/500 dilutions of the primary antibody, anti-PPARα (mouse-anti-human antibody, Millipore, code MAB 3890) were added to each tube, followed by a 15 minute incubation step at room temperature, in the dark. The secondary, fluorescent antibody (goat anti-mouse antibody, FITC-conjugated, R&D, code F0103B) was added as a 1/10 dilution, followed by a 15 minute incubation step at room temperature, in the dark (16, 17, 18). The study was designed to evaluate the difference in PPARα expression in leukocytes, between normal and MS- diagnosed subjects, therefore the use of isotype controls was not considered necessary. After the final wash, cells were transferred to 4.5 mL FACS tubes and analysed by flow cytometry using a FACS Aria III machine (Becton Dickinson, BD). Data acquisition was performed on a FACS Aria III machine (BD), and data analysis was carried out using the FlowJo software (TriStar Inc). We used the mean fluorescence intensity value (MFI) to evaluate the cell surface expression of these parameters**.** One example of analysis is depicted in Figure 1.

The gating procedure chosen is generally accepted in immunology and hematology, based on known patterns of cell population distribution on flow graphs (volume versus granularity) and it was consistently used for all subjects evaluated, for the entire duration of the study.

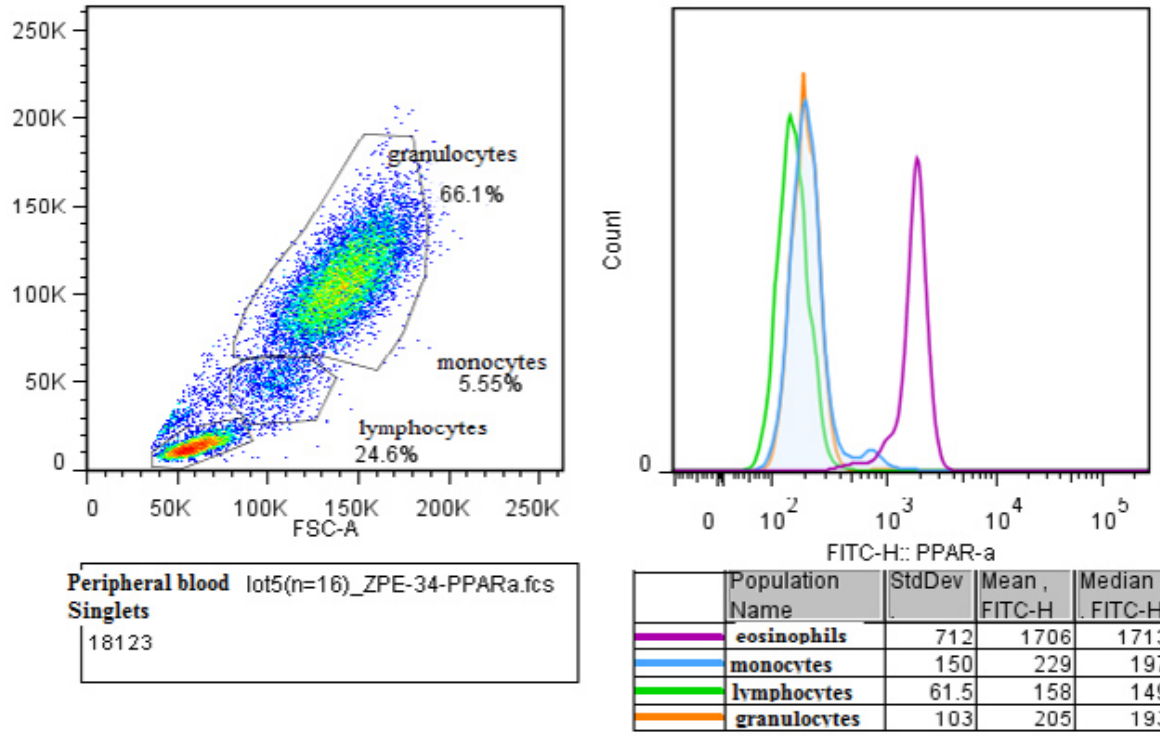
Mean Fluorescence intensity of CD36, as a measurement of relative level of its membrane expression, was detected by a conventional method for surface staining: 15 minutes incubation of 50 µL whole blood with 1:250 dilutions of monoclonal antibodies fluorochrome conjugated (CD36 – FITC, BD, code 561820 and CD14 - APC, BD, code 555399). The gating strategy for the Mo was based on known flow-cytometry profile: CD14 positivity *versus* intermediate side-scatter properties.

***Statistical analysis***

The statistical analysis was performed using SPSS 10 and Microsoft Office Excel 97-2003. We evaluated the differences between those two clusters of patients assessed for different variables taken into account. Using the t-test continuous variables were compared. Chi-square test was used to compare the categorical variables. A $p$-value of $<0.05$ was considered statistically significant. Pearson's correlation coefficient ($r$) was used to identify the statistical significance value when comparing the measured parameters for the two studied groups (19-21).

## Results

The distribution for the 57 patients taken into account was as follows: 45, 6% (n=26) presented MS (the MS group), 54, 4% (n=31) were negative for the syndrome, eligible for the control




| Population Name | StdDev | Mean , FITC-H | Median , FITC-H |
|---|---|---|---|
| eosinophils | 712 | 1706 | 1713 |
| monocytes | 150 | 229 | 197 |
| lymphocytes | 61.5 | 158 | 149 |
| granulocytes | 103 | 205 | 193 |

**Figure.1: Examples of flow cytometric analysis PPARα. Left diagram represents a forward scatter – side scatter distribution of leucocytes for a representative case; right displays the staining distribution (PPARα expression levels) of selected leucocyte populations (after eliminating doublet cells and gating).**

(non - MS) group. The patients were diagnosed according to IDF criteria.

When the two groups were compared, the statistical analysis revealed that WC ($p$<0.001), TG ($p$<0.001), glycemia ($p$<0.01), SBP ($p$<0.001), and DBP ($p$<0.001) positively correlated with the presence of MS, while HDL-chol was inversely correlated ($p$=0.001) with the presence of MS (Table 1).

PPARα and CD36 expression levels were analyzed in the MS and control groups in order to provide new parameters describing the molecular status of the cellular metabolic machinery.

When comparing MS- *versus* control subjects, a 16% significant decrease of the PPARα MFI was found in eosinophyls (Eo) (p<0.05; Figure 2A).

Also, when comparing MS *versus* control subjects, we noticed a 10% increase of CD36 MFI on Mo, that may suggest a consistent tendency ($p$ = 0.0729; Figure 2B). Furthermore, the staining intensity for PPARα in Eo was inversely correlated with the staining intensity for CD36 on Mo for both MS and non - MS subjects (Figure 2C).

There were no statistically significant differences in terms of the PPARα MFI on Mo or granulocytes or lymphocytes (Ly) between the two study groups. Nevertheless, the pattern of PPARα expression in Ly, Mo and granulocytes, positively correlated (r>0.5) one to each other, suggesting that, during the inflammatory response, there may be a common trigger for the PPARα expression in these cell types (Figure 3).

**Table 1. IDF Criteria of Metabolic Syndrome**

| | MS Lot (n= 26) | Control Lot (n=31) | p-value |
|---|---|---|---|
| Waist circumference *(cm) [1] | 99.18 ± 10.75 | 79.38 ± 9.38 | $p < 0.001$ |
| Glycemia (mg/dl) [1] | 118.93 ± 49.57 | 90.14 ± 8.82 | $p < 0.01$ |
| Triglycerides (mg/dl) [1] | 189.47 ± 79.20 | 87.66 ± 27.79 | $p < 0.001$ |
| HDL-cholesterol (mg/dl) [1] | 46.57 ± 10.26 | 57.71 ± 13.52 | $p = 0.001$ |
| Sistolic blood pressure (mmHg)[1] | 132.50 ± 11.15 | 111.93 ± 13.64 | $p < 0.001$ |
| Diastolic blood pressure (mmHg) [1] | 81.73 ± 9.48 | 72.09 ± 7.93 | $p < 0.001$ |
| Hypertension[2] | 69.23% | 16.12% | $p < 0.001$ |
| Diabetes mellitus[2] | 19.23% | 3.22% | $p < 0.001$ |
| Hypertriglyceridemia[2] | 80.76% | 12.90% | $p < 0.001$ |
| Reduced HDL-cholesterol[2] | 65.38% | 16.12% | $p < 0.001$ |

* - Waist circumference was ≥ 94 cm for men and ≥80 cm for women, according to the IDF definition of the metabolic syndrome

[1]- Data presented as mean ± standard deviation, *p* value for Student's *t* test.

[2]- Data presented as percentage, *p* value for Chi-square test.

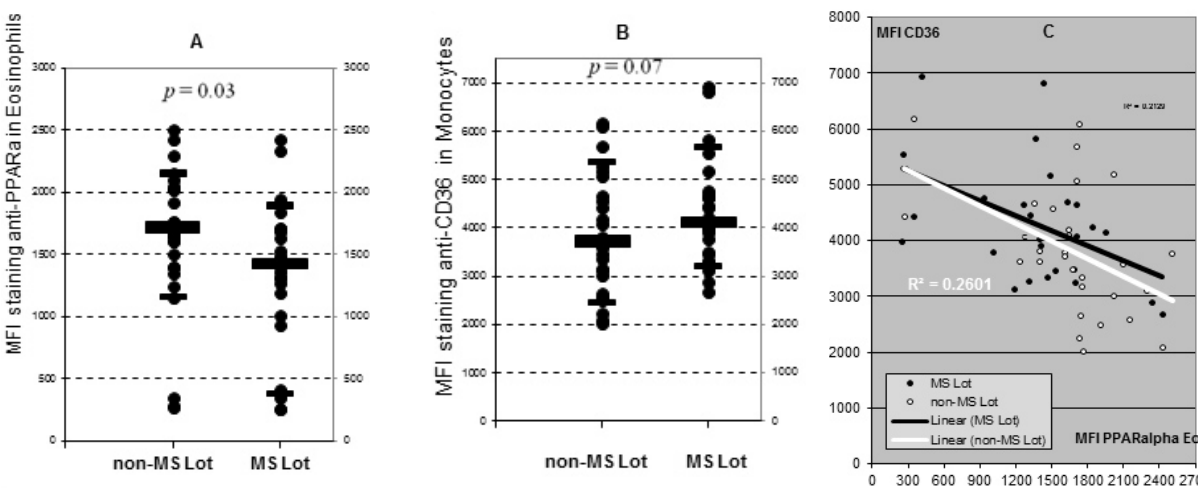



**Figure 2.Variation of MFI anti-PPARα in eosinophils (A) and MFI anti-CD36 in monocytes (B). The small lines locate the values of Percentile 90 and Percentile 10. The bold lines locate the values of Mediane and the dots represents individual values measured for each case. The graph C represents the correlations between MFI of PPARα in Eo versus MFI of CD36 in Mo.**

A significant, though weak, correlation of the PPARα MFI values in Eo *versus* Mo was found within the MS group ($r$ = 0.473, $p$ = 0.015), while within the control group this feature was absent ($r$ = 0.283, $p$ = 0.123).

WC was inversely correlated to PPARα expression in Mo for both, the non - MS ($r$ = - 0.474, $p$ = 0.007) and the MS Lot ($r$ = - 0.399, $p$= 0.044). Amongst normal subjects, the WC values were inversely correlated with the PPARα expression levels in the neutrophils ($r$ = - 0.404, $p$ = 0.024) and in the Ly ($r$ = - 0.495, $p$ = 0.005), while this was not the case of MS patients.

At the same time, statistically significant, moderate, positive Pearson correlations were calculated between WC and SBP ($p$ < 0.001), WC and DBP ($p$= 0.004), and WC and glycemia ($p$ = 0.003) among normal subjects. None of the above mentioned correlations is present in the MS lot. A moderate, positive correlation between WC and TG serum levels ($p$ = 0.042) was noticed within the MS group.

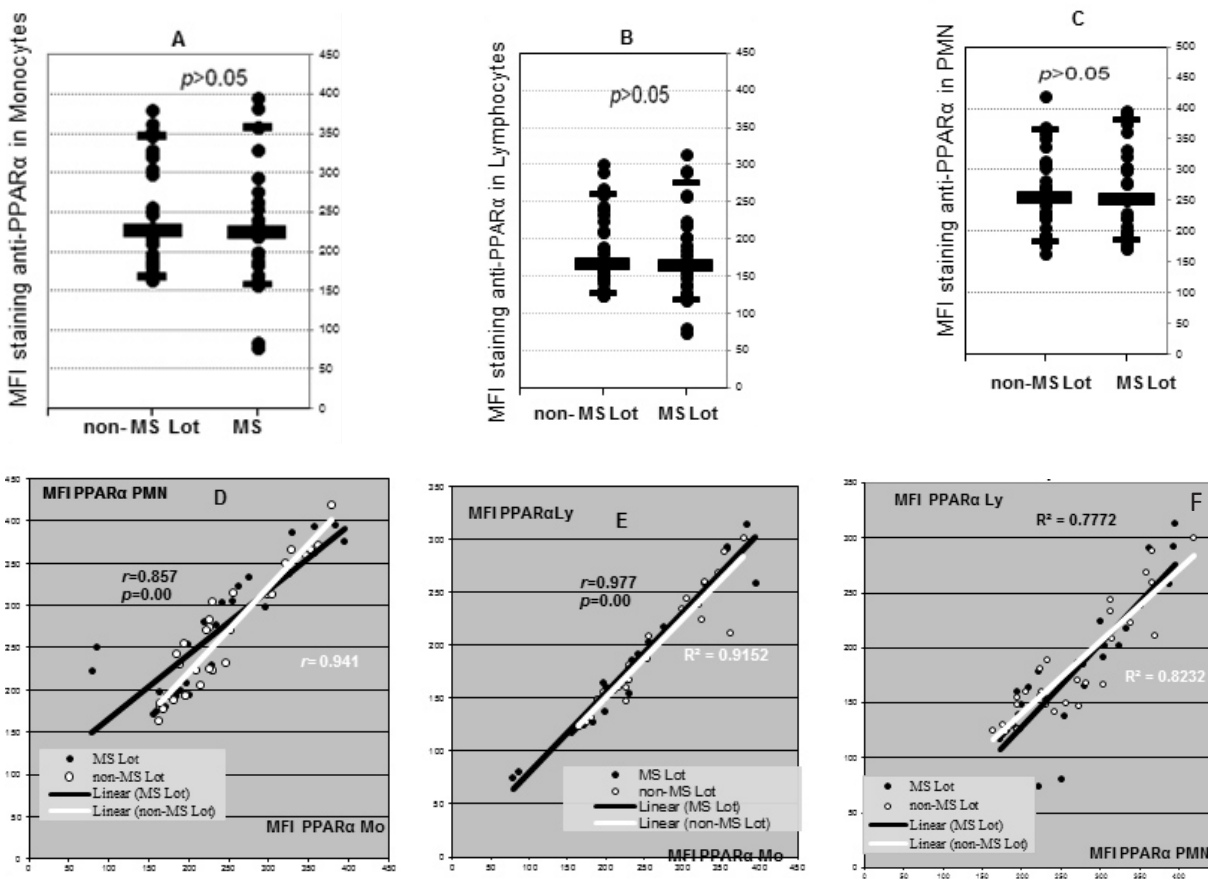



**Figure 3. Variation to PPARα receptor fluorescence intensity in monocytes (A), lymphocytes (B) and polymorphonuclears (C). In the graphs A, B and C, the small lines locate the values of Percentile 90 and Percentile 10. The bold lines locate the values of Mediane and the dots represents individual values measured. The correlations between PPARα in Mo vs PMN are shown in graph D, Mo vs Ly are represented in graph E and between PPARα in PMN vs Ly in graph F.**

Within the MS group, statistically significant, moderate, negative correlations are calculated when analysing the interdependence between PPARα expression levels in Mo ($p$=0.027) or Ly ($p$=0.031) and TG serum values. This relationship might be of high clinical interest since we also noticed the expected significant correlation between TG values and DBP values amongst the MS patients ($p$ = 0.002). However, when analysing the occurrence of correlations between MFI-PPARα in Mo and DBP values, we found no direct interdependence at all. No correlation can be found, too, when considering the distribution of DBP *versus* MFI-PPARα expression in Eo.

## Discussions

PPARα are nuclear receptors activated by natural ligands such as free fat acids or synthetic ligands such as fibrates, the later frequently used for dyslipidemia treatment (22). Acting like transcription factor, PPARα receptors control the expression of several genes involved in lipid metabolism and chronic inflammation (23). Their cellular expression is also subject to regulation under a complex control (9).

Our study measured the relative PPARα presence in leukocytes of MS patients and control subjects, utilizing a flow-cytometric method. Although the precise role of Eo in MS is uncertain, the Mo and Ly role in systemic inflammation and insulin resistance is highly acknowledged (8, 24).

Statistical significant differences in PPARα expression levels in Eo (Figure 2) were revealed in our study when comparing the MS and control groups, suggesting that PPARα are important in Eo involvement in insulin resistance and obesity.

Data derived from animal models is consistent with Eo involvement in MS. Reduced blood Eo values were associated in some mouse models with increase of the body weight and with insulin resistance, meanwhile increasing Eo count secondary to parasitosis or intense IL-5 stimulation is correlated with body weight loss and improved insulin resistance (25).

Concerning the extravascular space, Eo count in fat tissue is negatively correlated with fat deposition in animal models, furthermore suggesting that Eo are involved in the development of MS (26, 27). PPARα are expressed in Eo and they are able to regulate eosinophilia *in vivo* (demonstrated in a murine model of asthma) (28). These new exciting findings concerning the Eo worth must to be examined in MS patients, also, and clarify the extent of PPARα involvement in metabolism and inflammation.

Our study is the first to show that peripheral Eo PPARα protein expression in MS patients, as measured by flow cytometry, is significantly decreased, compared to control subjects. These observations suggest that the level of PPARα expressed in human Eo is related to MS. While we do believe that discrete alterations in PPARα expression and function in MS patients throughout the disease evolution and in various cell species, our resources were limited only to compare the status of this receptor in a lot of MS - diagnosed patients, *versus* non – MS individuals. Since we demonstrated significant differences in some cases, we do think that a real biological process is actually responsible. Furthermore, we believe that a relatively simple flow-cytometric methodology could be used to evaluate – at least under relative intensity staining (if not, with further refinements, in absolute values) – the level of PPARα expression in various circulating leukocytes. Such cell types are easily obtained (in contrast with bioptic materials, like endothelial or hepatic cells), and, if subject to the same homeostatic mechanisms controlling the PPARα translation like other cells in the body, such cells might provide information regarding the status of this molecular axis controlling the metabolism.

The study reliability is supported by highly significant statistical positive correlations among WC and BMI, BP, glycemia, elements belonging to the MS syndrome (increased WC connects to increased BMI, BP and glycemia).

The obtained results suggest that distinct molecular control mechanism operate in establishing the PPARα protein quantity in Eo *versus* other types of leukocytes (in MS patients), and that is consistent with reports describing the PPARα mRNA expression in leukocytes or other cell types (29, 30).

*Monocyte chemoattractant protein-1* (MCP-1 or CCL2) is implicated in pathogenesis of diseases characterised by monocytic infiltrates. The circulating levels of MCP-1 have been found significantly higher in human obese subjects, compared with normal subjects and the level of MCP-1 was related to the obesity-related parameters such as WC, BMI and CRP (31). Previous studies showed that PPARα activators had an anti-inflammatory effect on endothelial cells by blocking the induction of MCP-1 by CRP and glucose (32, 33). Moreover, since CCL2 can be delivered by adipocytes (34), both endothelial vascular walls and fat tissue can orchestrate the recruitment of circulating Mo in these spaces, suggesting that lowering PPARα expression relates to central obesity determinism. According to those data, the experimental study in a mouse model fed with oleylethanolamide (OEA) resulted in decreasing the body weight by activating PPARα in adipocytes, outcome that is not present in PPARA gene defective mice (35).

PPARα activation is associated to the modulation of lipid metabolism and PPARα-agonists are currently being used for dyslipidemia treatment (22). Since increased serum TG is associated in our study with increased DBP, the treatment with fibrates can be of use in decreasing DBP.

An atherogenetic prone genetic background is associated with increased cardiovascular disease and the involvement of certain allelic PPARα gene variants (36).

MS is associated with an increase of fatty acids, which will be converted into TG (7, 23, 37) and a chronic inflammatory status, which points our attention toward a PPARα receptor malfunction, leading to fatty acids beta oxidation and inflammation.

PPARα agonists treatments (fibrates) are efficient in lowering serum TG and decreased BP (38). This decrease of BP values is caused by the reduction of some inflammatory factors (IL-6, ICAM, VCAM-1), with roles in endothelial dysfunction and atherogenesis (7, 39). In our study, increased serum TG value in MS patients is correlated with increased DBP (unlike in the non-MS group), suggesting that a decline in PPARα expression and antiinflammatory activity in MS is consistent, and that its decline is attended by vascular endothelial malfunction.

Furthermore, the CD36 MFI values had the tendency to increase in the MS group, concordant with the reported CD36 atherogenic role (40, 41). CD36 expression has been proven to be increased in MS patients, certifying its importance during the pathogenesis of atherosclerosis. Based on such data, we believe that cellular expression levels for PPARα, CD36 and possible other connected molecules potentially involved in MS, such as CCL2, might be of help in early diagnosis and better understanding of this heterogeneous disease.

## Conclusions

The assessment of the relative expression of PPARα in circulating Eo and other leukocytes brings new insights into obesity and MS clinical evaluation. Simple flow-cytometric staining and measurement methods might grant access to the molecular homeostatic or lesion control systems deployed in various cellular types involved in the cardio-metabolic risk.

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## Statement on the potential conflict of interest

All authors declare no conflict of interest.

## Abreviations

µL - microlitres
$^0$C – Celsius degrees
APC - Allophycocyanin
ATP – Adult Treatment Panel
BD – Becton Dickinson
BMI – Body Mass index
BP – Blood Pressure
DBP – Diastolic blood pressure
DDW- Distilled Water
EDTA - Ethylenediaminetetraacetic acid
Eo – Eosinophil
FACS – Fluorescence-Activated Cell Sorting
FCS – Fetal Calf Serum
FITC – Fluorescein – 5 – isothiocyanate- protein conjugate
HDL- chol - High density lipoprotein cholesterol
ICAM- Intercellular adhesion molecule
IDF – International Diabetes Federation
IL- Interleukin
LDL – chol - Low density lipoprotein cholesterol
Ly – Lymphocyte
MCP- 1(CCL2) – monocyte chemoattractant protein-1
mL – millilitres
MFI – Mean fluorescence intensity
Mo –Monocyte
MS – Metabolic Syndrome
n – Number
Ox- LDL - Low density lipoprotein cholesterol
PMN – Polymorphonuclear leukocytes
PPAR – Peroxisome Proliferator -Activated Receptor
*r* – Pearson's correlation coefficient
SBP – Systolic blood pressure
SN – supernatant
TG – Triglycerides
VCAM – Vascular cell adhesion molecule
WC – Waist circumference

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