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Lead Guest Editor: Agata Stanek
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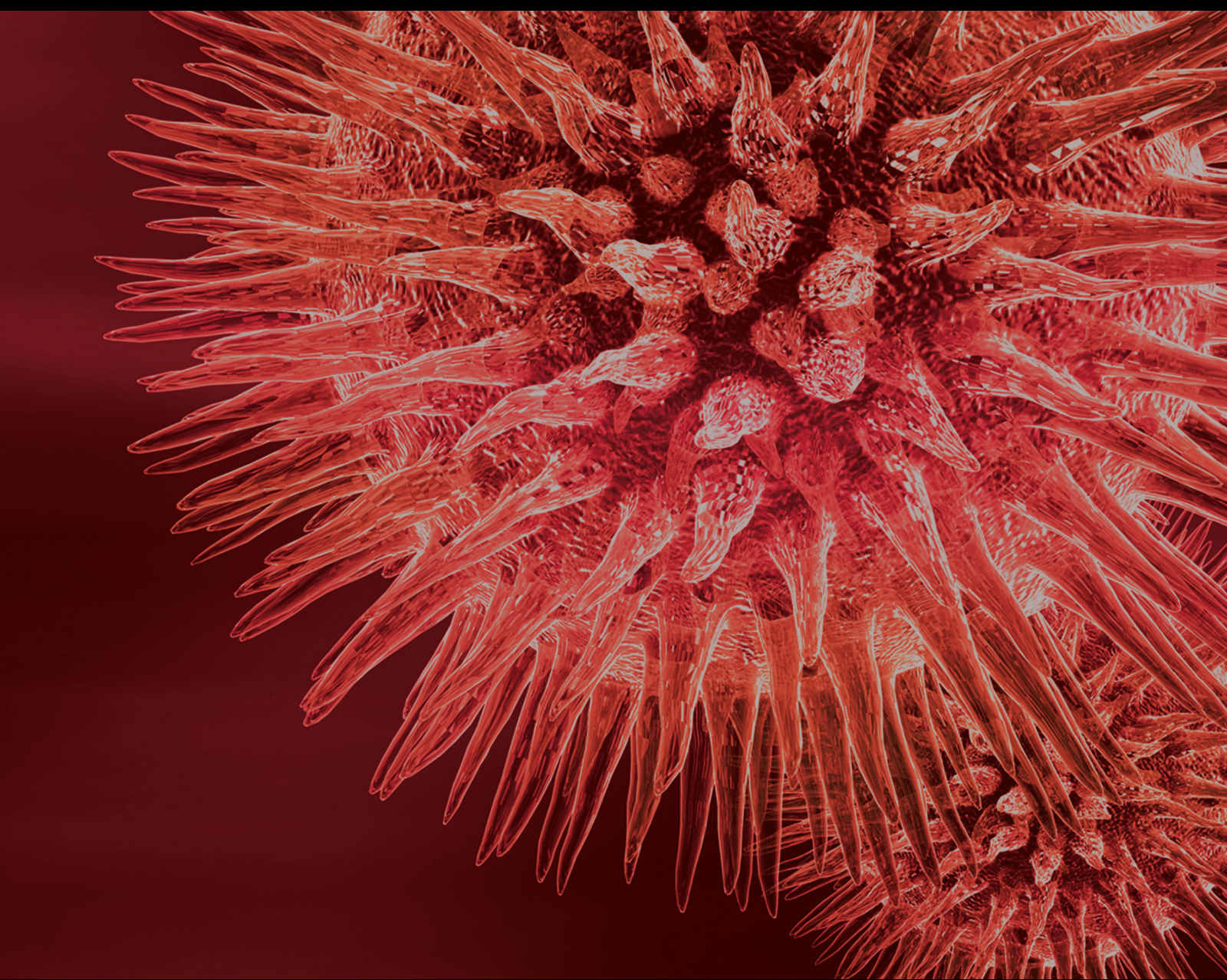

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Contents

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Editorial

The Role of Endothelium in Physiological and Pathological States: New Data

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Endothelium is the endocrine organ essential for maintenance of homeostasis in the entire body. Healthy endothelial cells regulate antioxidants, have anti-inflammatory and anticoagulant action, and, thus, control vascular relaxation and contraction, thrombogenesis, fibrinolysis, and platelet activation and inhibition. Maintaining the functional integrity of this complex organ is, therefore, critical for preserving blood flow and preventing thrombosis.

Endothelium dysfunction (ED) may result in the loss of important homeostatic functions, which, in turn, leads to various pathologies. ED has been observed in relation to ageing as well as in major lifestyle-related diseases, suggesting that endothelium dysfunction can serve as a means of identifying people for the purpose of preventing and treating various diseases. For example, ED is the initial stage in the pathogenesis of peripheral artery disease, cardiovascular diseases, chronic venous disease, chronic kidney failure, cancer, infectious diseases, and obesity.

This special issue comprises seven original research articles and two reviews, summarised in brief below, that delve into the important research being conducted on endothelial function.

The loss of endothelial integrity and function is a critical component of the onset of the adverse changes that result in cardiovascular disease. Injury of the endothelium is a consequence of haemodynamic (e.g., wall shear stress), chemical (e.g., low-density lipoprotein (LDL) cholesterol or glucose), or biologic factors (e.g., immune complexes). It is a known fact that mechanical factors, such as shear stress, are involved in the insult to the endothelial cells, but the level of this fluid drag force, which could damage the cells as well as the way they work, is still being studied. In the paper "Computing of Low Shear Stress-Driven Endothelial Gene Network Involved in Early Stages of Atherosclerotic Process", F. Vozzi et al. study the impact of different shear stress levels on endothelial gene expression in vitro using a laminar flow bioreactor. The authors show that different shear stress levels have a significant influence on the activity of the endothelial cells and their metabolic profile. They also demonstrate that lower shear stress promoted the proliferation properties of the examined cells by flow-induced gene expression modulation of some endothelial genes (upregulation for the genes particularly linked to the inflammation and apoptosis versus downregulation for the contrary acting genes).

Likewise, loss of endothelial integrity permits lipid infiltration, which forms the soft lipid of the atheromatous plaque. Many factors influence the development of atheromatous plague, many of which (so-called "modifiable risk factors") can be successfully treated. Amongst the applicable therapies are antiplatelet therapy and statin therapy. Both can result in very simple improvements, such as a decrease in platelet activity or a reduction in cholesterol level. However, the inflammatory theory of atherosclerosis resulted in the examination of these therapies' potential anti-inflammatory

effects as the usefulness and effectiveness of the drugs were assessed. In their paper "Simvastatin Effects on Inflammation and Platelet Activation Markers in Hypercholesterolemia", C. Barale et al. confirm that, in addition to its lipid-lowering effect, simvastatin acts as an anti-inflammatory and platelet-inhibiting agent. In their study, the simvastatin also improved the parameters of ED in patients with hypercholesterolemia. These changes were most likely a part of the pleiotropic effect attributed to the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors. The authors also suggest that a high LDL cholesterol level, which can be controlled by statins, could be responsible for platelet hyperactivity.

In their paper, P. A. Maranhão et al. demonstrate that ingestion of a high-fat meal involves the impairment of microvascular function in both obese and normal-weight women. High-fat meal intake can acutely impair microvascular function by inducing hyperinsulinemia, endothelial activation, and inflammation. This mechanism worsened ED in the obese women and induced the impairment of endothelial function in the normal-weight women. Due to the key role of ED in atherosclerosis, these findings may also explain the association between ingestion of a high-fat meal and atherosclerosis.

In their review paper, J. Gawrys et al. present the interactions between the cyclooxygenase metabolic pathway and the renin-angiotensin-aldosterone systems (RAAS) in the context of endothelial function balance disturbance. Based on available data, their brief review also summarises the data regarding usefulness and safety of the Acetylsalicylic acid (ASA) combination with drugs that act directly on RAAS.

In another review paper, M. Jakubowski et al. outline the current knowledge regarding the role of human platelet carbonic anhydrase II (CAII) in regulating platelet function. The paper also describes the consideration of this enzyme as a potential drug target and important pathophysiological chain in platelet-related disorders.

The known result of hyperglycaemia is tissue damage as a consequence of many mechanisms according to the polyol pathway, production of advanced glycation end products (AGEs), protein kinase C, and the hexosamine pathway, which are responsible for overproduction of the reactive oxygen species. Moreover, hyperglycaemia appears to be the major determinant of the diabetic microvascular complications' retinopathy, neuropathy, and nephropathy. Most significantly, kidney disease in patients with diabetes aggravates hypertension, which can further damage the kidneys and leads to end-stage renal disease. Because there is still no effective treatment for this complication, early diagnosis with treatment plays a crucial role in patient care. Furthermore, factors or molecules that can be detected in the early stages of nephropathy could lead to new opportunities for further treatment and may well also serve as a marker of endothelial damage, with all of its cardiovascular consequences. Such a factor appears to be the novel proangiogenic factor leucine-rich-α2-glycoprotein-1 (LRG1), which promotes abnormal angiogenesis within the renal glomerulus.

LRG1-induced abnormal angiogenesis resulting in glomerular fibrosis was observed in mice in a study by S. Haku et al. and is presented in the paper "Early Enhanced Leucine-Rich α-2-Glycoprotein-1 Expression in Glomerular Endothelial Cells of Type 2 Diabetic Nephropathy Model Mice". Because the glomerular LRG1 expression was increased before the vascular endothelial growth factor (VEGF) and vascular endothelial growth factor receptor-2 (VEGFR-2) increased, the authors suggest that glomerular LRG1 is an earlier marker of abnormal angiogenesis within the renal glomerulus than the mentioned classical growth factors.

For their part, M. A. Ortega et al. examined the behaviour of smooth muscle cells (SMCs) under hypoxic conditions: possible implications on the varicose vein endothelium. The authors share that the muscle cells of people with varicose veins showed levels of the studied markers (hypoxia-inducible factors 1α (HIF-1α), VEGF, transforming growth factor-β1 (TGF-β1), and endothelial nitric oxide synthase (eNOS) similar to normal cells subjected to hypoxia). They also demonstrate that, were the hypoxia to continue over the longer term, these cells would no longer have the capacity to react, and the factor that attempts to compensate for the hypoxia (EGLN3) would fail. Additionally, the authors presented totally different status for healthy subjects (controls) compared to the patients with venous insufficiency according to the VEGF level change. Apart from the difference in baseline VFGF concentration in normoxic conditions (higher level in patients with venous insufficiency), in the control group, the expression of VEGF raised in response to long- term hypoxia while in patients with varicose veins this factor's level decreased significantly.

The role of bone marrow-derived endothelial progenitor cells (EPCs) in monocrotaline-induced pulmonary arterial hypertension (PAH) is demonstrated in an original paper by R. Miao et al. In their study, it appeared that an increase in cytosolic free Ca^{2+} is identified as a trigger for promoting both proliferation and vasoconstriction of the pulmonary arterial smooth muscle cells. The balance of cytosolic calcium is controlled by the store-operated Ca^{2+} channels (SOC) in human EPCs, which is mediated by the Orai and the canonical transient receptor potential channels. The results of the study suggest that the association between the low expression of the major mediators of SOC and Ca^{2+} homeostasis may lead to PAH.

Endothelial function in children with acute lymphoblastic leukaemia (ALL) may predict he clinical outcome. A. Doroszko et al. demonstrate that high baseline vascular endothelial growth factor (VEGF) and soluble E-selectin levels, along with a significant increase in plasminogen activator inhibitor-1 (PAI-1) and low initial soluble intercellular adhesion molecule 1(sICAM-1) levels, are predictive of poor prognosis in ALL children.

The editors of the special issue, which is dedicated to the new insights to the endothelial function and its pathological states, strongly believe that the knowledge contained in this issue will mobilize many scientists to the further work as well as contributing to a better understanding human's physiology and pathology.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Acknowledgments

We would like to thank the editorial staff for the help during the whole extension of the preparation of the special edition and the kind reviewers that anonymously contributed to improving the manuscripts. All authors that chose to submit their works to this special edition are appreciated. We hope readers will enjoy the latest developments that are published in this special edition.

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Bahar Fazeli
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Research Article

Endothelial Function in Children with Acute Lymphoblastic Leukemia (ALL) May Reflect the Clinical Outcome

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Guest Editor: Agata Stanek

Endothelial dysfunction is a common feature of early complications of hemato-oncologic therapy. The aim of our study was to assess the profile of endothelial function at diagnosis time, then during initial treatment phase of acute lymphoblastic leukemia (ALL), and to verify the presence of its correlation with early clinical outcome (ECO). 28 ALL children and 18 healthy age-matched control ones were recruited. Study group was examined at baseline and at 33rd and 78th day of treatment. At each protocol step the endothelial function was assessed by measurement of sP-selectin (CD62-P), PAI-1(serpinE1), sE-selectin (CD62E), sICAM-1(sCD54), sVCAM-1(sCD106), and VEGF concentrations. Higher baseline sICAM-1 and sVCAM-1 levels and lower sP-selectin and VEGF were observed in children with ALL. sICAM-1, sVCAM-1, and sE-selectin levels were decreasing following the treatment with protocol I. Higher sE-selectin and lower baseline sICAM-1 levels were observed in children treated unsuccessfully. Lower PAI-1 levels were observed in children who survived. Higher baseline sE-selectin levels and lower sICAM-1 and VEGF were observed in children treated unsuccessfully. A decrease in sE-selectin and lower PAI-1 at the 78th day of therapy were associated with better ECO. High baseline VEGF and sE-selectin levels, significant increase in PAI-1, and low initial sICAM-1 levels are prognostics for poorer prognosis in the ALL children.

1. Introduction

Activation of vascular endothelium under pathological conditions is associated with an increased risk of death in numerous severe diseases [1–4]. Endothelial dysfunction (ED) is a common feature of many early complications of hemato-oncologic therapy which remain significant cause of morbidity and mortality despite the continuous optimization of treatment protocols [5]. Furthermore, their pathogenesis remains poorly understood.

Up to date, there are some established determinants of the endothelial cell injury development, but in a vast majority they are connected with chemotherapeutics (especially calcineurin inhibitors), methods used for conditioning (cytostatic, total body irradiation (TBI)), and certain aspects of the bone marrow transplant procedure itself

[6]. However, direct risk stratification associated with the present treatment is difficult due to the complexity and multiplicity of interactions, which can in different ways and magnitude generate dysfunction and structural damage to the endothelium. In our previous publication we showed that endothelial dysfunction belongs to pathologies observed in childhood ALL prior to treatment and it may result from increased plasma concentration of ADMA (asymmetric dimethylarginine). Additionally, at 78th day of treatment we observed both improvement in endothelial function and reduction of plasma ADMA concentration. Interestingly, it was accompanied by reduction of plasma prostacyclin concentration [7]. Recognizing endothelial dysfunction as a multifactorial disease, we intended to broaden in this paper the spectrum of analyses.

Under physiological condition there is a continuous balance in an interplay between chemokines acting pro- and antiaggregatory (such as PAI-1, sE-selectin, sP-selectin) as well as pro- and anti-inflammatory (i.e., TxB2, 6-keto-PGF-1α, sVCAM-1, sICAM-1) leading to the maintenance of vascular homeostasis and determining appropriate endothelial function [8]. Furthermore, the critical localization of endothelial cells between streaming blood and remaining components of vascular wall places them in a pathophysiological mainstream of cardiovascular consequences of hemato-oncological malignances.

ALL is an aggressive disease characterized by accumulation of immature malignant cells and the risk for relapse varies between patients and depends not only on genetic abnormalities, but also on growth factors action. An inverse relationship between blast proliferation and the magnitude of response to growth factors has been observed [9]. Furthermore, there are some reports stating that there are increased levels of soluble intercellular adhesion molecule-1 (sICAM-1) [10], soluble vascular cell adhesion molecule-1 (sVCAM-1), soluble E-selectin [11], thrombomodulin, and von Willebrand factor [12] in ALL children at the time of diagnosis, which indicates that ED may be present before the treatment begins.

To summarize, in this study we intended to clarify these relationships and verify the usefulness of analyzing selected markers of endothelial dysfunction for early risk stratification, as well as the ability to monitor the severity of complications. Hence, the aim of our observational study was to assess the profile of endothelial function at the diagnosis time as well as during initial phase of treatment and to verify the presence of its correlation with an early clinical outcome.

2. Material and Methods

All experiments were conducted and approved in accordance with the guidelines of the Bioethics Committee at Wroclaw Medical University and adhered to the principles of the Declaration of Helsinki and Title 45, US Code of Federal Regulations, Part 46, Protection of Human Subjects (revised November 13, 2001; effective December 13, 2001). All participants provided their informed consent which was followed by its written approval by a legal representative, as appropriate. The study and the written consent form had been approved by the Bioethics Committee at Wroclaw Medical University.

Table 1: Baseline characteristics of ALL children and control group.

Parameter	Study group (mean ± SEM)	Control group (mean ± SEM)	p
N	28	18	ns
Men (*n*)	12	6	ns
Age (*years*)	8.1 ± 1	11 ± 0.9	ns
ALT (U/L)	51.6 ± 32.3	17.7 ± 2.1	ns
AST (U/L)	49.2 ± 13	22.8 ± 2.4	0.02
Urea (mg/dL)	25.4 ± 1.6	21.3 ± 1.6	ns
Serum creatinine (mg/dL)	0.6 ± 0.0	0.6 ± 0.0	ns
Plasma glucose (mg/dL)	100 ± 5.9	80.4 ± 1.1	0.02
Detailed baseline characteristics of the study group			
	(mean ± SEM)		
CRP (mg/L)	23.2 ± 5.73		
Total bilirubin (mg/dL)	0.57 ± 0.11		
LDH (*μ*kat/L)	10.4 ± 1.6		
Total protein (g/dL)	6.8 ± 0.1		
Uric acid (mg/dL)	5.9 ± 0.5		
Sodium (mmol/L)	140 ± 0.4		
Potassium (mmol/L)	4.2 ± 0.1		

Abbreviations. AST: aspartate transaminase. ALT: alanine transaminase. hsCRP: C-reactive protein. LDH: lactate dehydrogenase. ns: not significant.

2.1. Patients and Controls. We have enrolled to our study 28 children with acute lymphoblastic leukemia and 18 healthy demographically matched children (Table 1).

The study group included children with established diagnosis of acute lymphoblastic leukemia treated strictly according to the guidelines published in ALL-IC BMF 2002 protocol [13] (Figure 1(b)) in the Department of Pediatric Bone Marrow Transplantation, Oncology and Hematology, Wroclaw Medical University (Wroclaw, Poland). Children were examined at the baseline (day of diagnosis), on the 33rd and 78th day of therapy. At all three points, blood samples had been obtained and endothelial function was assessed (Figure 1(a)).

ALL children were categorized into three risk groups (standard /n=8/, intermediate /n=14/, and high risk /n=6/). Independently of this fact, during the first 78 days all of them were treated in the same way with protocol I. The only difference was a doubled dose of daunorubicin in intermediate and high risk groups. The protocol M was implemented for the low-to-intermediate risk children, whereas the HR protocol was used for children with high risk. This was followed in turn by protocol II used in all the ALL children independently on the baseline risk (Figure 1). Remission was observed in all patients at the 33rd day of treatment.

The control group (n=18) was formed by healthy children hospitalized at general pediatrics ward (Wroclaw Medical University, Wroclaw, Poland) due to disorders that do not affect endothelial function. Controls were examined only once, since no changes in the profile of endothelial function were suspected.

2.2. Biochemical Tests. Blood was collected using the Sarstedt S-Monovette® system (Sarstedt AG & Co., Nümbrecht,

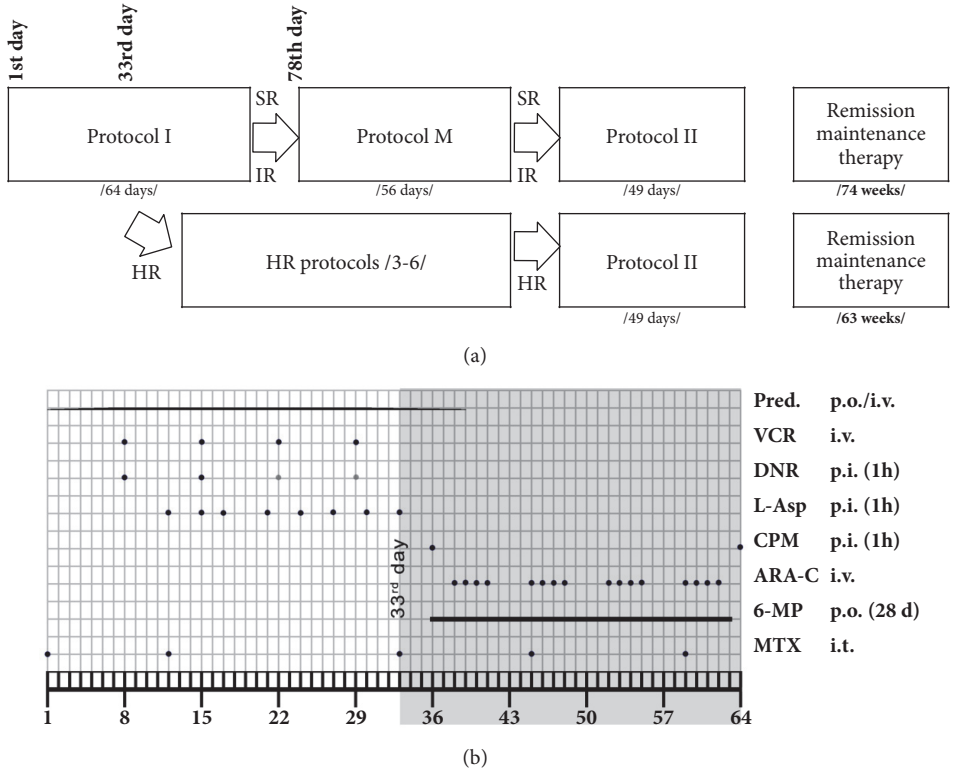

Figure 1: (a) Study design. (b) Treatment protocol. Protocol I in detail. Study covered the period of treatment common to all risk groups. *Abbreviations.* Pred, prednisone 60 mg/m^2/d; VCR, vincristine 1.5 mg/m^2/d; DNR, daunorubicin 30 mg/m^2/d; L-Asp, L-Asparaginase 5000 U/m^2/d; CPM, cyclophosphamide 1000 mg/m^2/d; ARA-C, cytarabine 75 mg/m^2/d; 6-MP, 6-mercaptopurine 60 mg/m^2/dl; MTX, methotrexate; p.o., per os; p.i., per infusion; i.t., intrathecal; d 1, day 1; d 33, day 33; SR, standard risk; IR, intermediate risk; HR, high risk.

Germany). Serum (7.5 mL) and EDTA plasma (9.0 mL; 1.6 mg-EDTA/ml of blood) were separated, immediately centrifuged (1000 x g for 15 minutes at 4°C), and frozen at −20°C for evaluation markers of endothelial activation.

2.3. Markers of Endothelial Activation. Plasma concentrations of sP-selectin (CD62-P) and PAI-1 (serpin E1) and serum concentrations of sE-selectin (CD62E), sICAM-1 (sCD54), sVCAM-1 (sCD106), and VEGF were determined by a sandwich enzyme immunoassay technique, using commercial ELISA kits (R&D Systems Europe. Ltd., 19 Barton Lane Abingdon Science Park OX14 3NB, United Kingdom) according to the manufacturer's instructions, kits catalogue numbers: BBE6, DSE100, DSLE00, DCD540, DVC00, DVE00, respectively. The coefficient of variation (*CV*), intra-assay %CV, was calculated as the ratio of the pooled standard deviation from all samples (each was analyzed in triplicate) and the overall mean and then multiplied by 100. Inter-assay %CV refers to assay-to-assay consistency that was calculated using the pooled standard deviation divided by the overall mean of all duplicated samples and then multiplied by 100. The intra-assay and inter-assay %CVs were, respectively, 5.2% and 7.6% for sP-selectin, 6.1% and 7.2% for PAI-1, 7.6% and 9.7% for sE-selectin, 5.3% and 7.1% for sICAM-1, 3.7% and 7.6% for sVCAM-15.8, and 7.2% for VEGF.

2.4. Other Biochemical Analyses. Concentrations of serum creatinine, urea, fasting plasma glucose, Aspartate transaminase (AST), Alanine transaminase (ALT), lactate dehydrogenase (LDH), high sensitivity C-reactive protein (hsCRP), potassium, and uric acid were measured using standard commercial laboratory assays.

2.5. Statistical Analysis. Data is expressed as the mean ± SEM. The differences between two continuous parameters were assessed using a Mann-Whitney U-test or a Student's t-test, followed by a Shapiro-Wilk test and Levene's test as appropriate. For comparison of more than two groups, an ANOVA followed by Tukey's test or a Friedman ANOVA test (for nonparametric statistics) was performed. Correlations between continuous variables were calculated using Spearman test. All calculations were made using Statistica 10.0 StatSoft® and the graphical representation of the data was performed using GraphPad 5 Prism®.

3. Results

3.1. General Characteristics of Groups. Both groups were similar regarding baseline demographic characteristics (Table 1). According to the expectations, there were significant differences related to the complete peripheral blood cell counts in children with ALL when compared to the control group (Figure 2(a)). Fasting glycemia was higher in ALL children,

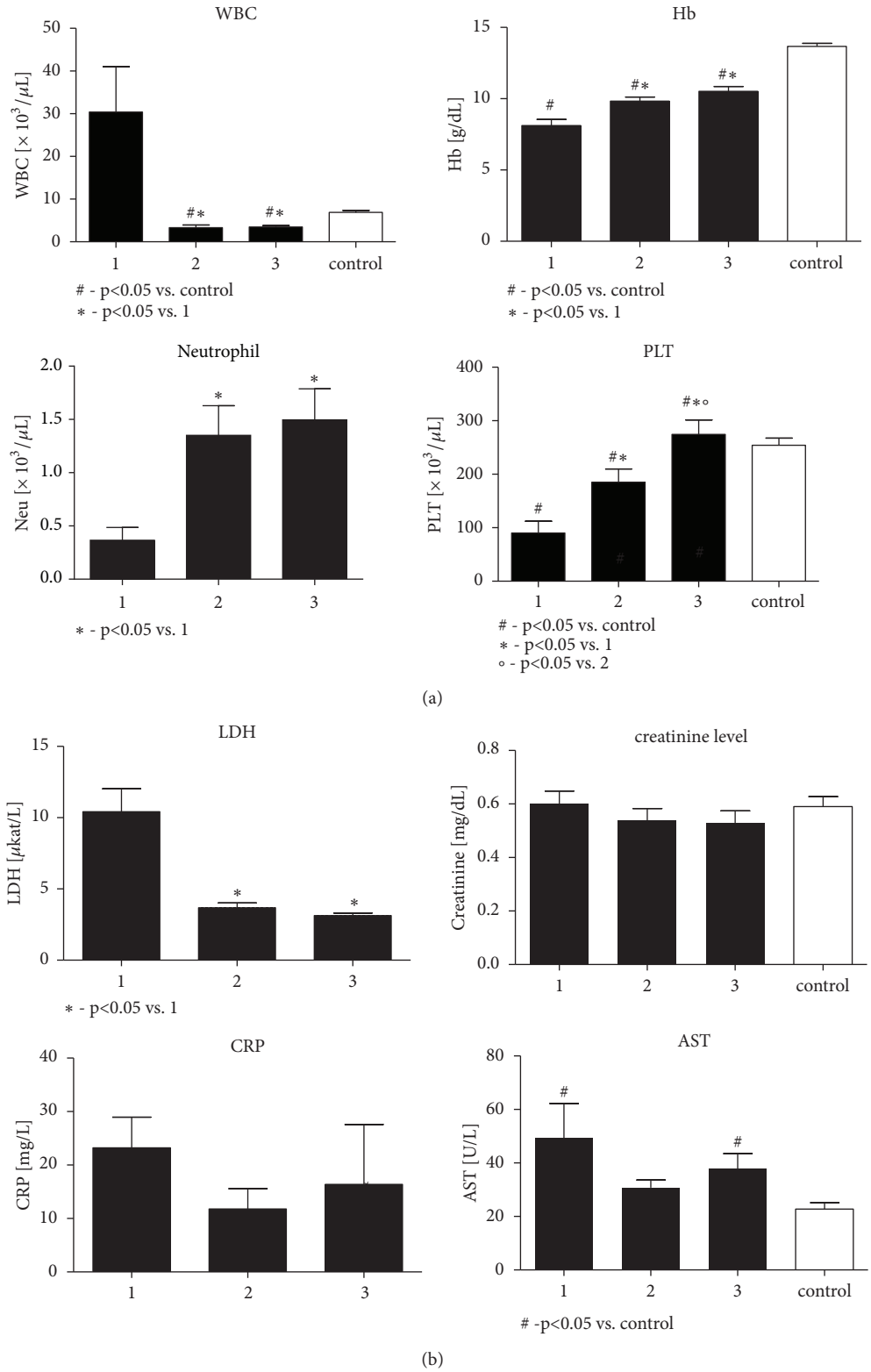

Figure 2: 1. day, 33. day, M (the protocol M beginning day)—points of evaluation in the study group. (a) Complete peripheral blood cell counts in children with ALL at particular steps of the study protocol and in the control group. (b) Basic biochemical characteristics of children with ALL at particular steps of the study protocol and of the control group.

as compared to the control at each step of the study protocol. The LDH levels were higher at the onset of ALL management; however in the course of treatment they recovered to the physiological normal range. Basic biochemistry results are presented in Figure 2(b) and Table 1.

3.2. Endothelial Activation Markers. Baseline sICAM-1 and sVCAM-1 levels, reflecting systemic inflammatory activation of endothelium, were significantly higher than those in control group. During treatment they significantly decreased, but still maintained higher levels compared to the control (Figure 3(a)). When analyzing the sICAM-1 and sVCAM-1 levels in the subgroups separated according to the risk, decreasing trends of the concentrations were observed only in the standard and intermediate risk groups (Figure 3(b)).

Baseline PAI-1 level in children with ALL was lower than that in controls. At 33rd day of observation the highest PAI-1 levels were observed compared to both control group and ALL children at other steps of the protocol (Figure 3(a)). These differences were significant in the whole study group, standard and intermediate risk group, but not in the high risk one. Furthermore, the high risk group was characterized by greater PAI-1 levels at the beginning of the protocol M (Figure 3(b)).

Analysis of the proaggregatory activation of endothelium, as assessed by the sE-selectin concentrations at the beginning and at the end of study time, did not differ significantly from control group. However, at 33rd day of treatment they were significantly lower when compared to both the control group and its baseline levels (Figure 3(a)). Additionally, baseline sE-selectin concentration in the high risk group was significantly higher as compared with the two remaining groups (Figure 3(b)). Significant decrease in the sE-selectin levels in the course of therapy was observed when compared both to the baseline values and to the control group.

Baseline VEGF and sP-selectin levels were significantly lower than values observed in further observation in whole study population and were also lower when compared to the control (Figure 3(a)). In ALL children, the sP-selectin concentrations at baseline were lower than during further treatment and also compared to control group. 33rd day levels (but not levels at the protocol M beginning) were significantly lower than ones in controls (Figure 3(a)).

3.3. Correlations with Cellular Lysis Markers. Statistically significant positive correlations between sVCAM-1 and AST as well as between sICAM-1 and uric acid levels were observed (Table 2).

3.4. Profile of Endothelial Function and a Short-Term Event-Free Survival (EFS). Analysis of the subgroups separated according to the outcome (a short-term survival in a 12-month follow-up from the date of enrollment to the study) has shown that higher baseline sE-selectin levels as well as marked increase in PAI-1 levels during the treatment were characterized with poor prognosis. Similarly, higher baseline VEGF levels and sICAM-1 were associated with poorer EFS (Figure 3(c)).

Table 2: Correlations between parameters assessed in the study group at baseline.

Parameters			Spearman's rank correlation coefficient	p
sVCAM-1	&	AST	0.51	0.01
sICAM-1	&	uric acid	0.44	0.03
sICAM-1	&	ADMA	0.61	0.01

Abbreviations. AST: aspartate transaminase, sVCAM-1: soluble vascular cell adhesion molecule 1, sICAM-1: soluble intercellular adhesion molecule 1.

4. Discussion

Our data broadens the scope of knowledge regarding pathophysiological abnormalities involved in endothelial dysfunction in ALL children. In this study we have demonstrated that proinflammatory and proaggregatory activation of endothelium is a common phenomenon in children with acute lymphoblastic leukemia. Moreover, serum cytokine and adhesion molecule profile in children with ALL may reflect important clinical characteristics related to both the risk profile and the outcome. Endothelial function in the study group at baseline was demonstrated to be partially impaired by endothelial inflammatory activation that accompanies the disease, which was reflected by elevated levels of sICAM-1 and sVCAM-1 [14]. Their concentrations are also positively correlated with cellular lysis markers (AST, uric acid) confirming thus that ED is a natural consequence of ALL. Cellular lysis resulting from increased cellular turnover in ALL seems thus to induce inflammation leading to development of endothelial dysfunction. In our previous work we showed that cellular lysis seems to cause also increased plasma concentration of ADMA, which is another established factor impairing endothelial function [7]. We verified that there is positive correlation between increase in concentration of ADMA and sVCAM-1 (r=0,61; p=0,01; Spearman's rank correlation coefficient; data not shown before).

In our study greater serum sICAM-1 levels, reflecting systemic inflammatory response, were associated with poor outcome and were noted in groups with greater than standard risk at baseline. This confirms the results presented in papers by Tacyildiz [10] and by Abdelrazik [15]. sICAM-1 plays a role in the immune response against tumor cells mediating the T cell cytotoxic response [16]. Aside from the fact that elevated levels of sICAM-1 represent an unfavorable prognostic marker in human neoplasias, sICAM-1 can be used to monitor the course of the disease [17] Similarly, increased serum levels of sVCAM-1 have been associated with tumor progression and metastatic potential in several solid malignancies. sVCAM-1 is normally expressed by bone marrow stromal cells, vascular endothelial cells, and follicular dendritic cells and its production is triggered by cytokines. In immunohistochemistry studies, VCAM-1 overexpression was found in biopsy material from acute leukemias [18]. Although the exact mechanism of release remains unknown, it is likely that sVCAM-1 is a product of enzymatic cleavage from endothelial or neoplastic cell surfaces induced by

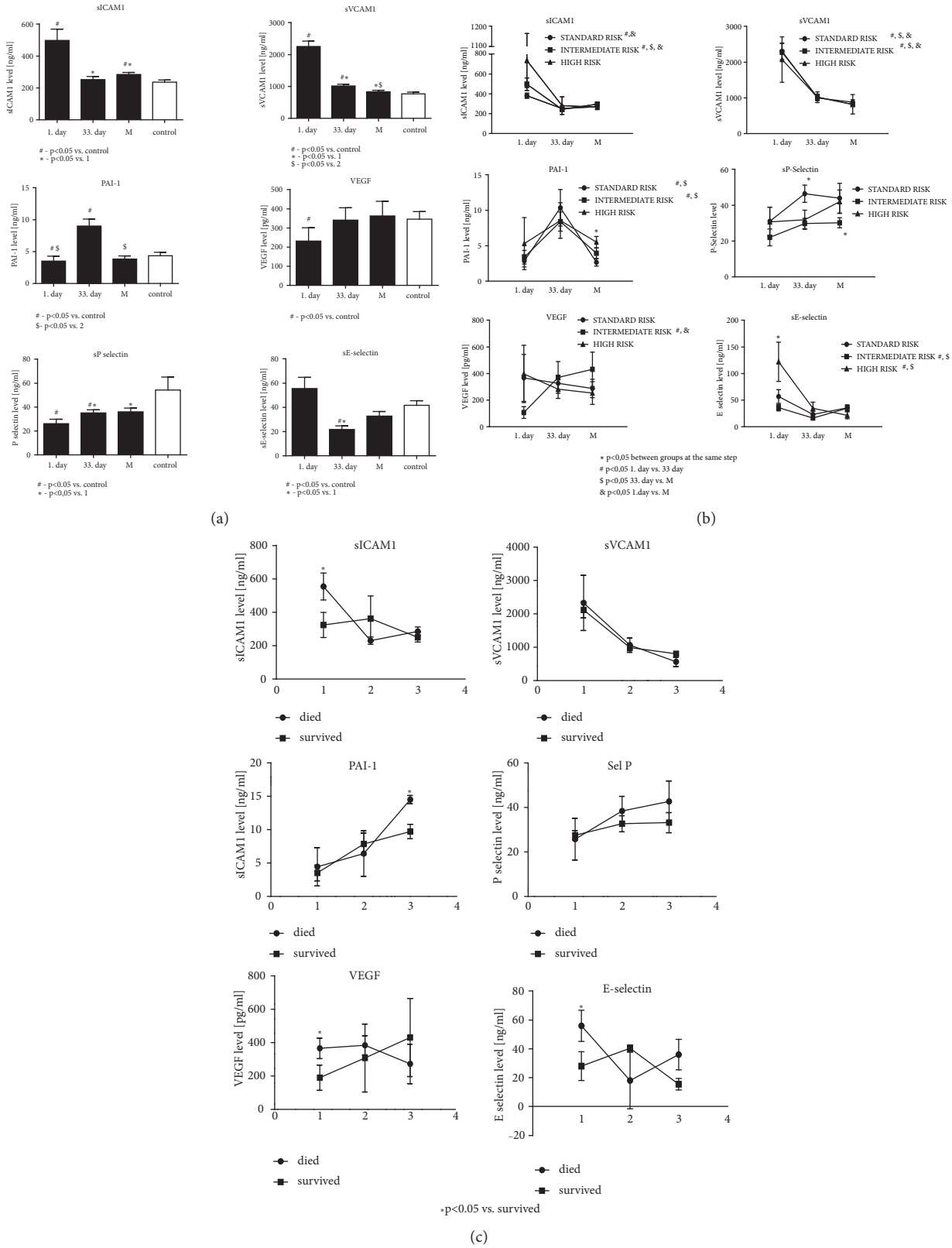

Figure 3: 1. day, 33. day, M (the protocol M beginning day)—points of evaluation in the study group. (a) Endothelial activation and systemic inflammation markers in children with ALL at particular steps of the study protocol and in the control group. (b) Analysis of endothelial activation and systemic inflammation markers in children with ALL assigned to the subgroups separated according to the risk stratification. (c) Analysis of endothelial activation and systemic inflammation markers in children with ALL assigned to the subgroups separated according to the short-term survival.

cytokines. Hence, determination of serum level of sICAM-1 and sVCAM-1 in ALL might represent an additional, but probably not independent, disease-associated marker for use in the clinical management of the children with acute lymphoblastic leukemia. Nonetheless, further studies are needed to establish if they could be used as a clinically relevant biomarkers in ALL management.

In this study we have shown for the first time the profile of changes in serum E-selection levels in pediatric ALL in relation to the risk group and outcome. Increased activation of endothelial cells at the onset of therapy (as reflected in sE-selectin concentrations) was associated in our study with worse prognosis and was present in children qualified to the high risk group at the beginning of therapeutic protocol. Other studies have shown similar effect of elevated serum E-selectin levels but either in other hematological malignancies [19] or in context of severe immunological complications of ALL [20].

Another marker of the ALL course considered in this paper is an endothelial plasminogen activator inhibitor (PAI-1). Plasminogen activator (PA) is implicated in solid tumor growth, invasion, and metastasis. However, little is known about its role in leukemia. Some studies have shown that PA and PAI-1 are synthesized by leukemic cells, thus pointing out that plasminogen activation may contribute to the invasive behavior of these cells [21]. In clinical studies, high levels of PAI-1, induced by corticosteroid treatment, lead to fibrinolysis suppression through inhibition of tissue plasminogen activator and thrombosis promotion[22]. As a result increased intraosseous venous pressure blocks blood flow to the femoral head and culminates in hypoxic bone death or osteonecrosis. It affects up to one-third of patients treated for ALL [23]. In our study, greater PAI-1 levels at the beginning of the protocol M were both associated with poorer prognosis (assessed by event-free survival /EFS/) and more predominant in children qualified to the high risk group at the beginning of therapeutic protocol. Hence, we postulate that the analysis of an increase in PAI-1 levels in children in the course of treatment could reflect increased risk for thrombotic complications. Nonetheless, verification of the usefulness of PAI-1 level in prospective follow-up of children with ALL during the therapy requires future large-number clinical trial.

Vascular Endothelial Growth Factor (VEGF) has been demonstrated to regulate the physiological and pathological angiogenesis. Its expression is associated with proliferation of neoplastic cells, their invasion and metastasis [24] VEGF may also play some role in pathogenesis of leukemia, since deregulation of VEGF expression and signaling pathways are observed in ALL [25]. Our data confirms studies by Kalra et al. [26] and Dincaslan et al. [27], demonstrating that untreated children with ALL have significantly lower VEGF concentration than controls and that, at the end of the induction therapy, VEGF increases to levels similar to controls [26, 27]. Hence, we confirm also the hypothesis that since VEGF is expressed in normal hematopoietic cells, the renewal of normal hematopoiesis in the course of therapy may explain the increase in VEGF levels and be a predictor of good response to the chemotherapy. This confirms also the data from the study by Avramis et. al stating that high VEGF levels in induction are a predictive marker for EFS [28].

As observed in our study proaggregatory and proinflammatory endothelial activation may lead in the long-term observation to the earlier onset of cardiovascular disorders in this subpopulation. Hence, we believe that the cardiovascular follow-up of the grown up childhood cancer survivors should be recommended.

5. Conclusions

Proinflammatory and proaggregatory activation of endothelium is a common phenomenon in children with acute lymphoblastic leukemia. High baseline serum levels of VEGF, high initial sICAM-1 and sE-selectin, and significant increase in PAI-1 levels are prognostics for poorer prognosis in a short-term observation in children treated for ALL. Evaluation of serum cytokine and adhesion molecule profile in children with ALL may reflect important clinical characteristics. However, further, larger clinical studies are needed in order to establish their exact usefulness in predicting the short- and long-term outcome in children with acute lymphoblastic leukemia.

Data Availability

All the original data of the study used to support the findings are included within the article. The raw data used to support the findings of this study are restricted by the Bioethics Committee at Wroclaw Medical University in order to protect the patients' privacy.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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Research Article

Early Enhanced Leucine-Rich α-2-Glycoprotein-1 Expression in Glomerular Endothelial Cells of Type 2 Diabetic Nephropathy Model Mice

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Abnormal angiogenesis plays a major role in the development of early stage diabetic nephropathy. Vascular endothelial growth factor (VEGF) is a classical proangiogenic factor that regulates abnormal glomerular angiogenesis linked to glomerular hypertrophy in the early stage of diabetic nephropathy. Leucine-rich α-2-glycoprotein-1 (LRG1) was recently reported as a novel proangiogenic factor that is expressed in endothelial cells and promotes angiogenesis by modulating the transforming growth factor-β signaling pathway. However, the pathophysiology of LRG1 in diabetic nephropathy remains largely unknown. In the present study, we investigated intrarenal expression of the novel proangiogenic factor LRG1 in diabetic *db/db* mice by immunohistochemistry and a laser capture microdissection method during the development of diabetic nephropathy. We hypothesized that glomerular LRG1 expression is increased earlier than VEGF expression under conditions of pathological angiogenesis in the early stage of diabetic nephropathy. Thus, we compared glomerular expression of VEGF and LRG1 in diabetic *db/db* mice at 16 and 24 weeks of age. At 16 weeks, diabetic *db/db* mice exhibited glomerular hypertrophy with abnormal angiogenesis characterized by endothelial cell proliferation, which was concomitant with an increase in LRG1 expression of glomerular endothelial cells. However, glomerular VEGF expression was not increased at this early stage. At 24 weeks, the features of early diabetic nephropathy in *db/db* mice had developed further, along with further enhanced glomerular LRG1 expression. At this late stage, glomerular VEGF and fibrosis-related-gene expression was also significantly increased compared with nondiabetic *db/m* mice. These results suggest that LRG1 plays a pivotal role in the initial development of diabetic nephropathy by promoting abnormal angiogenesis, thereby suggesting that LRG1 is a potential preemptive therapeutic target of diabetic nephropathy.

1. Introduction

Diabetic nephropathy has become a leading cause of end stage renal disease (ESRD) worldwide [1, 2]. Despite treatment by inhibition of the renin angiotensin system and tight glycemic control, the risk of ESRD remains high. Diabetic nephropathy is diagnosed at the early stage by detection of microalbuminuria. However, early prevention of diabetic nephropathy progression remains challenging. Therefore, understanding the pathogenesis of early stage diabetic nephropathy and developing methods to control its progression are important issues.

Abnormal angiogenesis plays a major role in the development of early stage diabetic nephropathy [3, 4]. It is associated with glomerular hypertrophy and urinary albumin excretion [5–7]. Morphological changes in capillaries, such as elongation and an increased number, contribute to glomerular hypertrophy [3, 4]. In these aberrant vessels, the endothelial cells are swollen and immature, leading to increased vascular permeability [5, 8, 9].

Vascular endothelial growth factor (VEGF) is a critical regulator of abnormal angiogenesis, and its glomerular expression is involved in the pathogenesis of early stage diabetic nephropathy [10–12]. VEGF is mainly expressed in podocytes, and its receptor, vascular endothelial growth factor receptor-2 (VEGFR-2), is expressed in glomerular endothelial cells [13]. VEGF-VEGFR-2 signaling is upregulated in the diabetic glomerulus, which leads to abnormal angiogenesis and endothelial cell proliferation [14].

Very recently, Leucine-rich α-2-glycoprotein-1 (LRG1), a novel proangiogenic factor expressed in endothelial cells [15], has been reported to be involved in the development of diabetic nephropathy. Transcriptomic and *in vitro* analyses revealed that LRG1 in glomerular endothelial cells is one of the key regulators in the development of abnormal angiogenesis at the early stage of diabetic nephropathy [16]. However, the pathophysiology of LRG1 in diabetic nephropathy is still largely unknown.

The aim of this study was to compare glomerular expression of the classical proangiogenic factor VEGF and novel proangiogenic factor LRG1 in the early stage of diabetic nephropathy. We investigated glomerular expression of VEGF and LRG1 in a mouse model of diabetes (C57BL/KsJ-db/dbJcl; *db/db* mouse) and compared the changes in expression at 16 and 24 weeks of age to evaluate their association with diabetic nephropathy development.

2. Materials and Methods

2.1. Animals. This study was performed in accordance with the National Institutes of Health guidelines for the use of experimental animals. All animal experiments were reviewed and approved by the Animal Studies Committee of Yokohama City University. Male *db/db* mice and their age-matched nondiabetic *db/m* (C57BL/KsJ) littermates were purchased from CLEA Japan. The mice were housed in a controlled environment with a 12-hour light-dark cycle at 25°C. The mice were allowed free access to food and water. Mice were fed a standard diet (0.3% NaCl, 3.6 kcal/g, and 13.3% energy as fat; Oriental MF, Oriental Yeast Co, Ltd.). The mice were sacrificed at 16 and 24 weeks of age.

2.2. Biochemical Assays. Blood glucose was measured in blood obtained by tail vein puncture. Blood samples were also collected by cardiac puncture when mice were sacrificed in a fed state as described previously [17, 18]. Whole blood was centrifuged at 800 × *g* for 10 minutes at 4°C to separate the plasma. The resulting plasma was stored at -80°C until use. Plasma creatinine and urinary creatinine were measured using an autoanalyzer (Hitachi 7180; Hitachi, Tokyo, Japan). Urinary albumin was measured by an ELISA kit (Fujifilm Wako Shibayagi, Gunma, Japan).

2.3. Metabolic Cage Analysis. To collect urine, metabolic cage analysis was performed as described previously [19, 20]. Mice were provided with free access to tap water and fed a standard diet.

2.4. Histological and Immunohistochemical Analyses. Kidneys were fixed with 4% paraformaldehyde and embedded in paraffin. Sections of 4 μm in thickness were stained with periodic acid Schiff (PAS). Immunohistochemistry was performed as described previously [17, 18, 21]. Briefly, the paraffin-embedded sections were dewaxed and rehydrated. Antigen retrieval was performed by microwave heating. The sections were treated for 60 minutes with 10% normal goat serum in phosphate-buffered saline and blocked for endogenous biotin activity using an AVIDIN/BIOTIN Blocking kit (Vector Laboratories, CA, USA). The sections were then incubated with one of the following antibodies: (1) anti-LRG1 antibody (TAKARA, Japan) diluted at 1:100 or (2) anti-CD34 antibody (BD Pharmingen, Japan) diluted at 1:100. The sections were incubated for 60 minutes with biotinylated goat anti-rabbit IgG (Nichirei Corporation, Tokyo, Japan), blocked for endogenous peroxidase activity by incubation with 0.3% H_2O_2 for 20 minutes, treated for 30 minutes with streptavidin and biotinylated peroxidase (DAKO, Heidelberg, Germany), and then exposed to hematoxylin, dehydrated, and mounted. To evaluate the glomerular area, 25 glomeruli per mice were measured and averaged. All images were acquired using a BZ-9000 microscope (Keyence).

2.5. Real-Time Quantitative PCR Analysis. Total RNA was extracted from the kidney with ISOGEN (Nippon Gene, Tokyo, Japan), and cDNA was synthesized using the SuperScript III First-Strand System (Invitrogen, Carlsbad, CA, USA). Real-time quantitative PCR (RT-qPCR) was performed by incubating the reverse transcription product with TaqMan Universal PCR Master Mix and TaqMan probes (Applied Biosystems, Foster City, CA, USA), as described previously [22, 23]. mRNA levels were normalized to 18S rRNA as a control.

2.6. Laser Capture Microdissection and Subsequent RT-qPCR Analysis. Laser capture microdissection (LMD) was performed using a Leica LMD System (LMD 6000), as described previously [24, 25]. Briefly, formalin-fixed, paraffin-embedded tissues were cut into 10 μm-thick sections, mounted on polyethylene terephthalate membrane slides, and stained with hematoxylin-eosin. Next, renal glomeruli were microdissected using the LMD 6000 laser microdissection microscope. In total, 350 glomeruli were microdissected from the renal cortex per mouse. Total RNA was extracted from microdissected tissue using the RNeasy FFPE Kit (Qiagen, Hilden, Germany). cDNA was synthesized using the SuperScript III First-Strand System and applied to TaqMan RT-qPCR analysis.

Table 1: Body weight, blood glucose, kidney weight, plasma creatinine, and albuminuria of *db/db* and *db/m mice*.

Variable	16W		24W	
	db/m (n=6)	*db/db* (n=6)	*db/m* (n=4)	*db/db* (n=4)
Body weight (g)	29.2 ± 1.3	48.2 ± 0.7∗∗	37.0 ± 0.5	52.2 ± 4.8∗
Blood glucose (mg/dl)	134.8 ± 5.8	>600∗∗	130 ± 6.7	574 ± 26∗∗
Kidney weight (mg)	182.3 ± 6.7	217.3 ± 8.5∗∗	225.0 ± 6.7	263.3 ± 5.8∗∗
Plasma creatinine (mg/dl)	0.12 ± 0.22	0.11 ± 0.1	0.08 ± 0.01	0.09 ± 0.02
Albuminuria (mg/mg·Cr)	0.25 ± 0.02	0.67 ± 0.16∗∗	0.04 ± 0.01	1.05 ± 0.53∗

All values are means ± SEM.
$*P < 0.05$, $**P < 0.01$, vs *db/m* mice at the same age, unpaired *t*-test.

2.7. Statistical Analysis. Statistical analysis was performed using GraphPad Prism software (GraphPad Software, La Jolla, CA, USA). All quantitative data are expressed as the mean ± SEM. Differences were analyzed using the unpaired Student's *t*-test. Values of $P < 0.05$ were considered as statistically significant.

3. Results

3.1. Characteristics of 16- and 24-Week-Old db/db Mice. Body weight, blood glucose, kidney weight, and the albuminuria level were significantly higher in *db/db* mice compared with *db/m* mice at 16 weeks of age (Table 1). At 24 weeks of age, body weight, blood glucose, kidney weight, and the albuminuria level were significantly increased in *db/db* mice compared with *db/m* mice. However, serum creatinine levels were identical in *db/db* and *db/m* mice at both 16 and 24 weeks of age (Table 1).

3.2. Glomerular Hypertrophy with Endothelial Cell Proliferation in db/db Mice. At 16 weeks of age, *db/db* mice exhibited a significantly larger glomerular area compared with *db/m* mice ($3974.0 \pm 117.6\ \mu m^2$ versus $5359.0 \pm 202.3\ \mu m^2$, $P < 0.001$) (Figure 1(a)). At 24 weeks of age, the glomerular area had increased further in *db/db* mice compared with *db/m* mice ($3542.2 \pm 95.2\ \mu m^2$ versus $5689.2 \pm 182.5\ \mu m^2$, $P < 0.0001$) (Figure 1(b)). We next examined CD34 immunostaining, as an endothelial cell marker, in the glomeruli of *db/db* and *db/m* mice. The immunostaining analysis showed enhancement of glomerular CD34 expression in *db/db* mice compared with *db/m* mice at 16 weeks of age (Figure 1(c)), and this trend became robust at 24 weeks of age (Figure 1(d)). These increases in glomerular endothelial cells indicated that abnormal angiogenesis had occurred in the kidneys of diabetic *db/db* mice [3, 26]. To confirm the increase of glomerular CD34 expression in *db/db* mice, we next examined CD34 mRNA expression in glomeruli of *db/db* and *db/m* mice using an LMD method. Glomerular CD34 mRNA expression was significantly increased in *db/db* mice compared with *db/m* mice at 16 and 24 weeks of age (Figures 1(e) and 1(f)). These results indicate that diabetic *db/db* mice exhibit glomerular hypertrophy with endothelial cell proliferation from the early stage of their lives, and these features are exacerbated along with aging.

3.3. Enhanced LRG1 Immunostaining in Glomerular Endothelial Cells of db/db Mice at 16 Weeks of Age. We next examined LRG1 expression and distribution in the renal cortex of *db/db* and *db/m* mice at 16 weeks of age by immunohistochemical analysis. The results showed that *db/db* mice exhibited an increase in LRG1 expression of glomerular endothelial cells, whereas LRG1 was weakly expressed in glomerular endothelial cells of *db/m* mice (Figures 2(a) and 2(b)). LRG1 was also weakly expressed in tubular epithelial cells of renal tubules in *db/db* and *db/m* mice. However, LRG1 expression was not found in podocytes of *db/db* and *db/m* mice. These results indicate that an increase in LRG1 expression of glomerular endothelial cells might be associated with the development of diabetic nephropathy in *db/db* mice.

3.4. Enhanced Glomerular LRG1 mRNA Expression in db/db Mice at 16 Weeks of Age. We next examined gene expression in glomeruli of *db/db* and *db/m* mice at 16 weeks of age using the LMD method. Glomerular LRG1 mRNA expression was significantly increased by approximately 2.5-fold in *db/db* mice compared with *db/m* mice (1.0 ± 0.24 versus 2.4 ± 0.57, $P < 0.05$) (Figure 3(a)). Intriguingly, glomerular mRNA expression levels of VEGF and its main receptor, VEGFR-2, which are key mediators of abnormal angiogenesis and glomerular hypertrophy, were not increased in *db/db* mice compared with *db/m* mice (Figures 3(b) and 3(c)). Furthermore, glomerular mRNA expression levels of fibrosis-related genes, such as transforming growth factor-β (TGF-β), Collagen type IV (Collagen IV), and plasminogen activator inhibitor-1 (PAI-1), were identical in *db/db* and *db/m* mice (Figures 3(d)–3(f)). Since LRG1 directly binds to the TGF-β accessory receptor endoglin (ENG), and then the complex binds to TGF-β receptor-II (TβRII), we also examined glomerular mRNA expression of ENG and TβRII in *db/db* and *db/m* mice. However, there was no significant difference in glomerular mRNA expression levels of ENG and TβRII between *db/db* and *db/m* mice (Figures 3(g) and 3(h)). These results showed that the increase in LRG1 expression preceded the increase in expression of other angiogenesis- and fibrosis-related genes in the glomerulus of diabetic *db/db* mice.

3.5. Enhanced LRG1 Immunostaining in Glomerular Endothelial Cells of db/db Mice at 24 Weeks of Age. We next examined LRG1 expression in the renal cortex of *db/db* and *db/m* mice

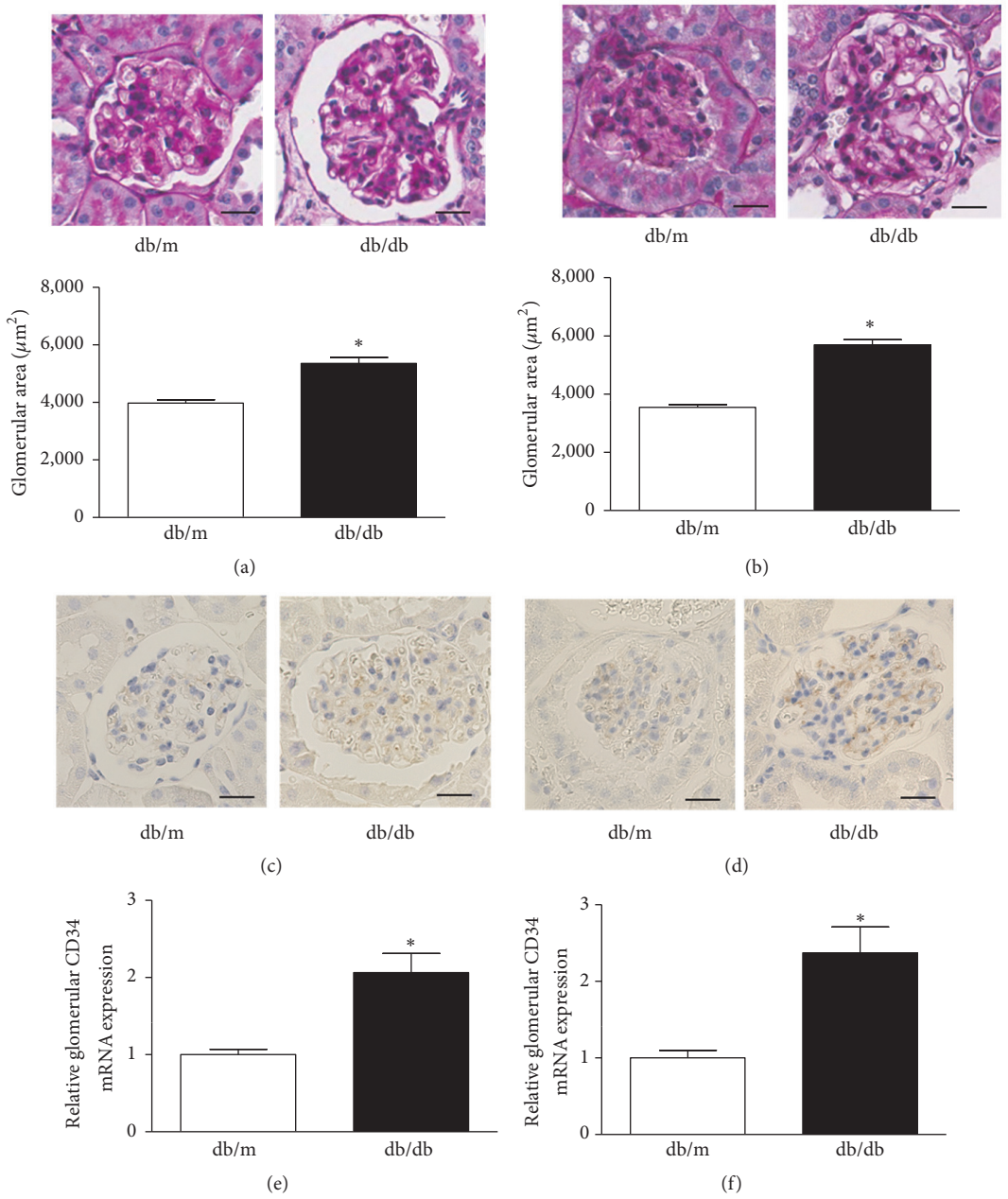

Figure 1: **Glomerular hypertrophy with endothelial cell proliferation in *db/db* mice.** Representative image of PAS staining in glomeruli of *db/m* and *db/db* mice at (a) 16 weeks and (b) 24 weeks of age. The glomerular area was increased in *db/db* mice at both ages (25 glomeruli per mice were measured and averaged). Values are expressed as the mean ± SEM (n = 4–6 in each group). $*P < 0.05$ versus *db/m*, unpaired *t*-test. Original magnification: ×200, bars = 20 μm. Representative image of CD34 immunostaining at (c) 16 weeks and (d) 24 weeks of age. Quantitative analysis of CD34 in 350 glomeruli identified by an LMD method at (e) 16 weeks and (f) 24 weeks of age. Values are expressed as the mean ± SEM (n = 4–6 in each group). $*P < 0.05$ versus *db/m*, unpaired *t*-test.

at 24 weeks of age by immunohistochemical analysis. The results showed that LRG1 expression was further enhanced in glomerular endothelial cells of *db/db* mice compared with *db/m* mice at 24 weeks of age (Figures 4(a) and 4(b)).

3.6. Increases in Glomerular LRG1, VEGF, and Fibrosis-Related Gene Expression of db/db Mice at 24 Weeks of Age. Finally, we examined gene expression in glomeruli of *db/db* and *db/m* mice at 24 weeks of age. Glomerular LRG1 mRNA expression was further increased by approximately 3.5-fold in *db/db* mice compared with *db/m* mice (1.0 ± 0.21 versus 3.40 ± 0.48, $P < 0.01$) (Figure 5(a)). Glomerular VEGF and VEGFR-2 mRNA expression was also significantly increased in *db/db* mice compared with *db/m* mice (Figures 5(b) and 5(c)). In

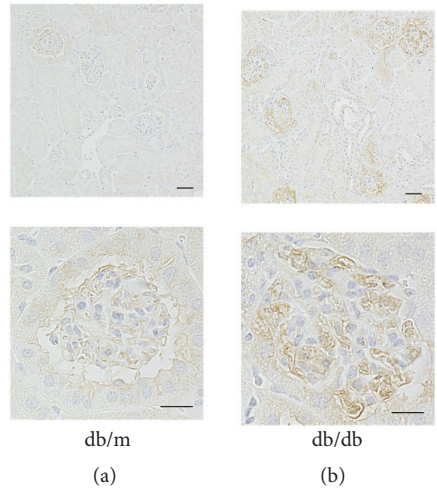

FIGURE 2: **Enhanced LRG1 immunostaining in glomerular endothelial cells of *db/db* mice at 16 weeks of age.** Representative image of LRG1 immunostaining at 16 weeks of age in (a) *db/m* and (b) *db/db* mice. Lower panels are higher magnifications of immunohistochemical staining for LRG1 in glomerular endothelial cells. Upper panels: original magnification: ×40, bars = 20 μm. Lower panels: original magnification: ×400, bars = 40 μm.

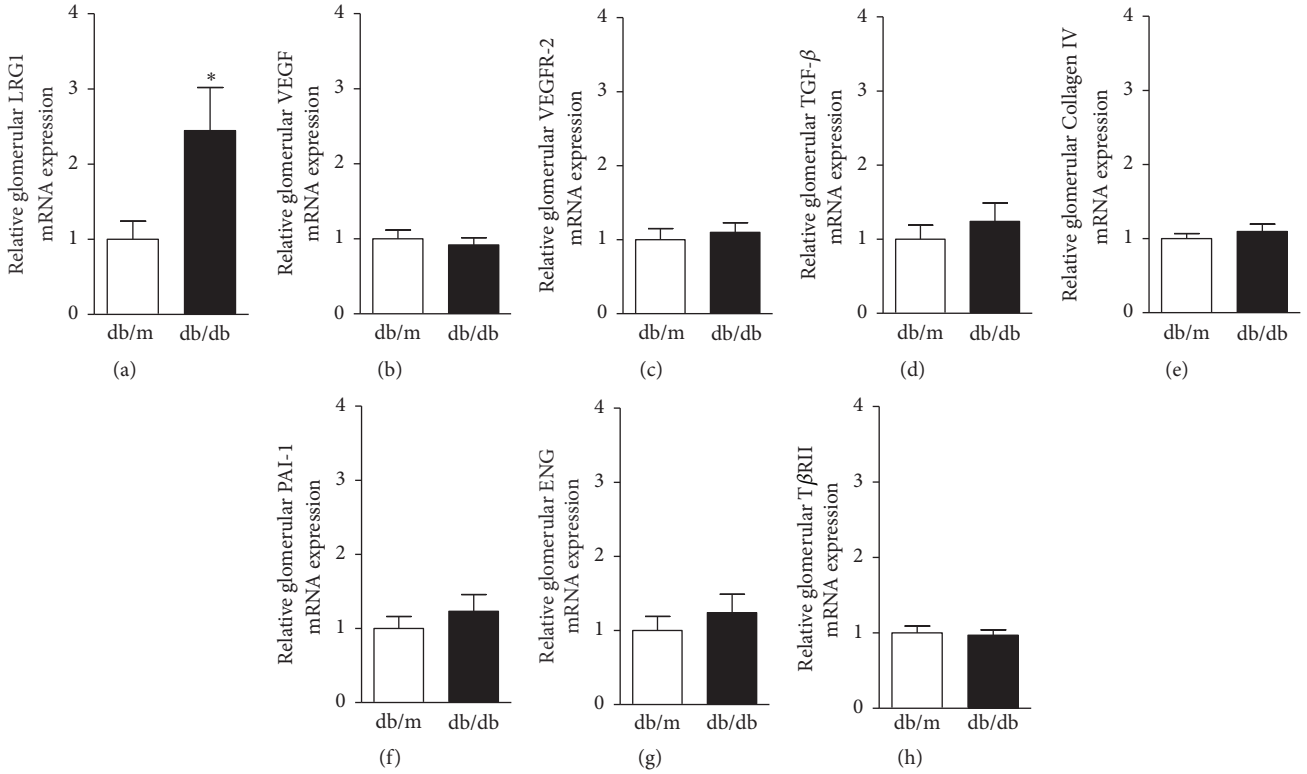

FIGURE 3: **Enhanced glomerular LRG1 mRNA expression in *db/db* mice at 16 weeks of age.** Quantitative analysis of angiogenesis- and fibrosis-related gene expression in glomeruli identified by the LMD method in *db/m* and *db/db* mice at 16 weeks of age. (a) LRG1, (b) VEGF, (c) VEGFR-2, (d) TFG-β, (e) Collagen IV, (f) PAI-1, (g) ENG, and (h) TβRII. Values are expressed as the mean ± SEM (n = 4–6 in each group). $*P < 0.05$, versus *db/m* mice, unpaired *t*-test.

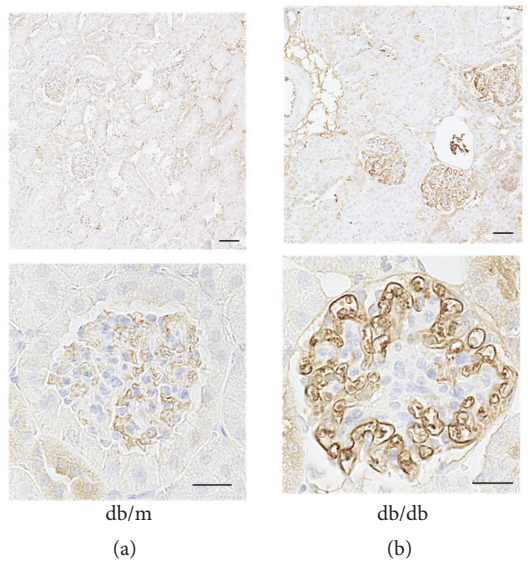

FIGURE 4: **Enhanced LRG1 immunostaining in glomerular endothelial cells of *db/db* mice at 24 weeks of age.** Representative image of LRG1 immunostaining at 24 weeks of age in (a) *db/m* and (b) *db/db* mice. Lower panels represent higher magnifications of immunohistochemical staining for LRG1 in glomerular endothelial cells. Upper panels: original magnification: ×40, bars = 20 μm. Lower panels: original magnification: ×400, bars = 40 μm.

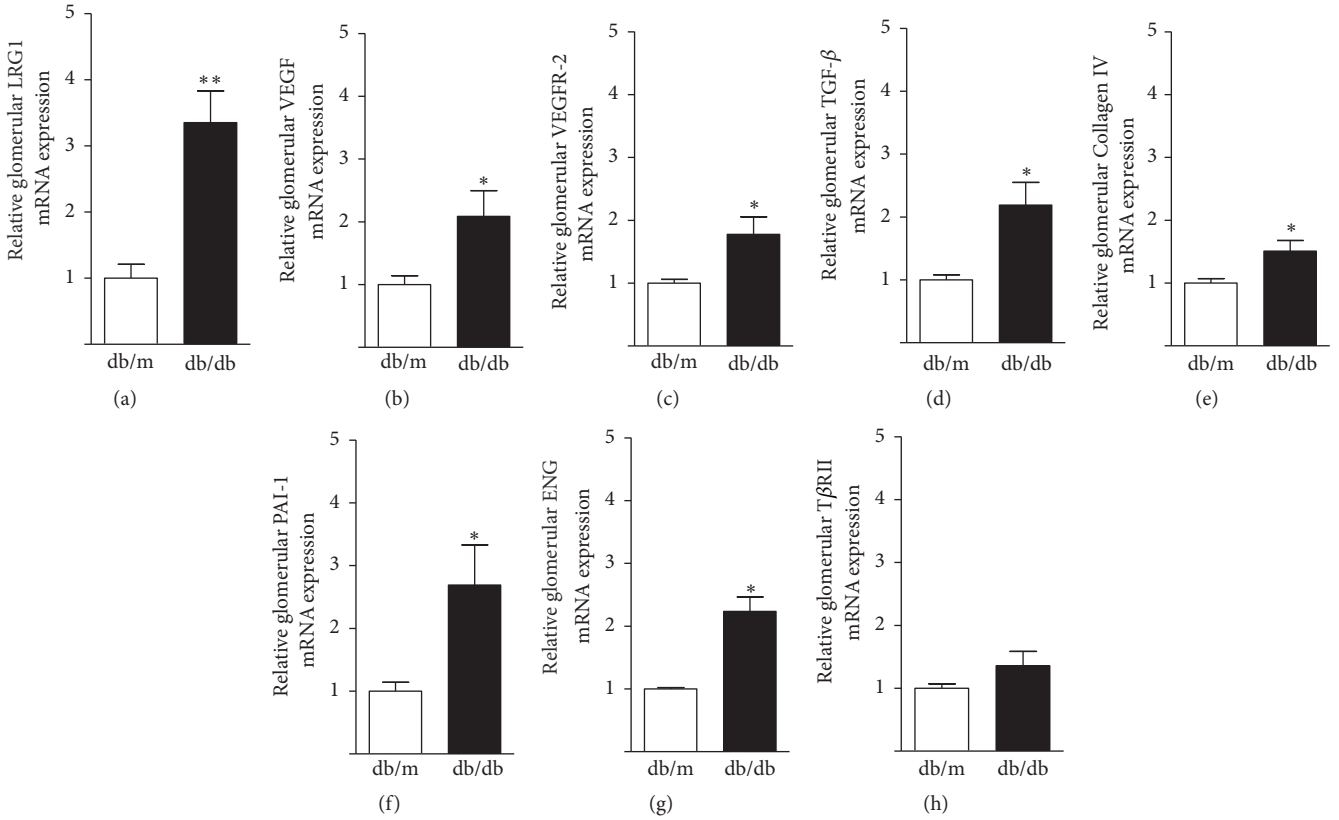

FIGURE 5: **Enhanced glomerular LRG1, VEGF, and fibrosis-related gene expression in *db/db* mice at 24 weeks of age.** Quantitative analysis of angiogenesis- and fibrosis-related gene expression in glomeruli identified by the LMD method in *db/m* and *db/db* mice at 24 weeks of age. (a) LRG1, (b) VEGF, (c) VEGFR-2, (d) TGF-β, (e) Collagen IV, (f) PAI-1, (g) ENG, and (h) TβRII. Values are expressed as the mean ± SEM (n = 4–6 in each group). $*P < 0.05$, $**P < 0.01$ versus *db/m* mice, unpaired *t*-test.

addition, the glomerular expression levels of fibrosis-related genes, such as TGF-β, Collagen IV, and PAI-1, were significantly increased in *db/db* mice compared with *db/m* mice (Figures 5(d)–5(f)). Similarly, glomerular mRNA expression levels of ENG and TβRII were significantly increased in *db/db* mice compared with *db/m* mice (Figures 5(g) and 5(h)). These results indicated that diabetic nephropathy had developed further after the preceding increase in glomerular LRG1 expression of *db/db* mice.

4. Discussion

In the present study, we investigated the intrarenal expression and distribution of a novel proangiogenic factor, LRG1, along with the development of early stage diabetic nephropathy. Our most important finding is that the increase in LRG1 expression of glomerular endothelial cells precedes the increase in VEGF expression, which is another proangiogenic factor involved in the development of diabetic nephropathy. This finding suggests that LRG1 plays a major role in the initial development of diabetic nephropathy by promoting abnormal angiogenesis and glomerular hypertrophy.

In diabetic *db/db* mice at 16 and 24 weeks of age, they exhibited moderate increases in albuminuria levels and glomerular hypertrophy without nodular glomerulosclerosis. These data indicate that our diabetic mice represented the features of early stage diabetic nephropathy, as reported previously [27]. Notably, at 16 weeks of age, glomerular LRG1 expression in diabetic mice was elevated despite glomerular VEGF expression being not increased yet. Considering the results indicating that the glomerular volume and endothelial cells were significantly increased in diabetic mice at this early age, abnormal angiogenesis would already exist in the glomerulus. Thus, there is the possibility that the abnormal angiogenesis observed in the very early stage of diabetic nephropathy in *db/db* mice was attributed to LRG1.

The interaction between LRG1 and VEGF is still unclear, but some studies have shown a possible link between these two factors. In the retina of LRG1 knockout mice, VEGF expression is significantly decreased compared with wild-type mice [15]. In colorectal cancer cells, LRG1 directly induces VEGF expression and promotes tumor angiogenesis [28]. However, LRG1 also exerts proangiogenic effects independently of the VEGF signaling pathway. Double blockade of LRG1 and VEGF suppresses angiogenesis more efficiently than single blockade in the mouse model of choroidal neovascularization [15]. In this study, we demonstrated for the first time that glomerular LRG1 expression was increased earlier than VEGF and VEGFR-2 expression under the conditions of pathological angiogenesis in the early stage of diabetic nephropathy. This finding suggests that glomerular LRG1 is involved in the pathogenesis of abnormal angiogenesis independently of the VEGF signaling pathway, at least in the very early stage of diabetic nephropathy.

Although LRG1 binds directly to ENG and activates the ALK1-Smad1/5/8 pathway for angiogenesis, the TGF-β-mediated Smad2/3 pathway is subsequently activated as a counterbalance against angiogenesis to induce quiescence in the endothelium [29, 30]. This vicious pathological cycle of angiogenesis and quiescence finally leads to glomerular fibrosis in diabetic nephropathy [31–34]. In our study, 24-week-old *db/db* mice exhibited upregulation of glomerular fibrosis-related genes, which was concomitant with glomerular hypertrophy and endothelial cell proliferation. It is assumed that the LRG1-induced abnormal angiogenesis observed in 16-week-old *db/db* mice resultantly led to glomerular fibrosis at the later stage.

Our study did not demonstrate whether the increased glomerular LRG1 expression actually caused the abnormal angiogenesis in diabetic *db/db* mice. Therefore, the functional role of LRG1 in abnormal angiogenesis in the early stage of diabetic nephropathy must be investigated further using LRG1 transgenic and knockout mice. Nevertheless, the findings of the present study provide important information regarding the pathogenesis of abnormal angiogenesis in early stage diabetic nephropathy, suggesting that LRG1 is a novel preemptive therapeutic target in diabetic nephropathy.

5. Conclusions

Diabetic *db/db* mice exhibited glomerular hypertrophy with abnormal angiogenesis characterized by endothelial cell proliferation at 16 weeks of age, concomitant with an increase in LRG1 expression of glomerular endothelial cells. However, glomerular VEGF expression was not increased at this early stage. The features of early diabetic nephropathy in *db/db* mice had developed further, along with further enhanced glomerular LRG1 expression at 24 weeks of age. Glomerular VEGF and fibrosis-related-gene expression was also significantly increased at this late stage compared with nondiabetic *db/m* mice. These data demonstrate that LRG1 might be pivotal for the initial development of diabetic nephropathy by promoting abnormal angiogenesis.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare no conflicts of interest.

Authors' Contributions

Sona haku and Hiromichi Wakui designed and conducted the study. Sona haku, Hiromichi Wakui, and Kengo Azushima wrote the manuscript. Sona haku, Hiromichi Wakui, Kengo Azushima, Kotaro Haruhara, Sho Kinguchi, Kohji Ohki, Kazushi Uneda, Ryu Kobayashi, Miyuki Matsuda, Takahiro Yamaji, Takayuki Yamada, Shintaro Minegishi, Tomoaki Ishigami, Akio Yamashita, and Kenichi Ohashi performed the experiments. Sona haku, Hiromichi Wakui, and Kotaro Haruhara analyzed the data. Akio Yamashita, Kenichi Ohashi, and Kouichi Tamura supervised the study. All authors approved the final manuscript.

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Research Article

Metabolic Changes Induced by High-Fat Meal Evoke Different Microvascular Responses in Accordance with Adiposity Status

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Background. Frequently, ingestion of lipids exceeds our daily requirements and constantly exposes humans to circulating lipid overload which may lead to endothelial dysfunction (ED), the earliest marker of atherosclerosis. Nailfold videocapillaroscopy (NVC) technique can detect ED on microcirculation. Using NVC, we aimed to demonstrate if metabolic alterations evoked by high-fat meals can act differently on microvascular endothelial reactivity in lean and women with obesity. *Methods.* Women, aged between 19 and 40 years, were allocated to control group (CG) and with obesity group (OBG) and were subjected to blood analysis for determination of glucose, total cholesterol (TC), triglycerides (TG), and low-density lipoprotein cholesterol (LDL-c) and high-density lipoprotein cholesterol (HDL-c) and NVC evaluation at fasting and 30, 60, 120, and 180-min after high-fat meal ingestion. NVC technique evaluated microvascular reactivity through the following variables: red blood cell velocity (RBCV) at rest and after 1-min ischemia (maximal red blood cell velocity, $RBCV_{max}$) and time taken to reach it ($TRBCV_{max}$). A P value ≤ 0.05 was considered significant. *Results.* High-fat meal promoted a two-phase response in both groups: one until 60-min, associated with glucose and insulin levels, and the other after 120-min, associated with TG levels. Significant differences between groups were observed concerning insulin and HDL-c concentrations only at fasting and TC, TG, and LDL-c levels in all-time points. Regarding microvascular reactivity, RBCV, $RBCV_{max}$, and $TRBCV_{max}$ were significantly different in OBG at 30-min compared to baseline. $RBCV_{max}$ and $TRBCV_{max}$ were significantly different in CG at 30-min and 60-min comparing to fasting. In all-time points, OBG presented RBCV, $RBCV_{max}$, and $TRBCV_{max}$ significantly different in comparison to CG. *Conclusion.* High-fat meal worsened ED on microcirculation in women with obesity and induced impairment of endothelial function in lean ones, reinforcing the association between high-fat meal and atherosclerosis.

1. Introduction

Cardiovascular diseases (CVD) are associated with atherosclerosis and are the main cause of death worldwide [1], and their incidence tends to rise as a consequence of world epidemic of obesity [2], which is known to be a risk factor for atherosclerosis [3].

Changes in plasma lipoprotein concentrations are a well-established cause of increased CVD risk [4–6]. However, it does not explain why individuals with normal fasting levels of plasma lipids develop atherosclerosis. Zilversmit [7] was the first to hypothesize that atherogenesis is a postprandial phenomenon since humans spend most part of their days in the postprandial state. Moreover, frequently the ingestion of lipids exceeds the real requirements of the organism, and as a consequence, we are regularly exposed to an overload of circulating lipids [8, 9]. This lipid overload leads to excessive formation of damaging reactive oxygen species

(ROS), leukocyte activation, endothelial dysfunction, and foam cell formation [10–13]. The consumption of high-fat meals can turn acute postprandial microvascular endothelial dysfunction into a chronic vascular disease [14].

High-fat diet modifies critical protective functions of endothelium, and as a consequence, the endothelium becomes dysfunctional with a greater tendency for the development of atherosclerosis [15]. The most remarkable feature of endothelial dysfunction is the impairment of endothelium-dependent vasodilatation due to decreased NO bioavailability due to decreased NO synthesis or increased depletion by ROS [16, 17].

Since endothelial dysfunction is considered an early marker of the atherosclerotic process, it is crucial to assess its earliest manifestations in micro- and macrovessels. Recently, we have demonstrated that nailfold videocapillaroscopy (NVC) can detect the first derangements of microvascular function in women with obesity even before the appearance of obesity-related comorbidities [18]. Thus, using this technique, we aimed to demonstrate that metabolic alterations elicited by high-fat meal ingestion can impair microvascular function. We additionally investigated if lean women have different microvascular responses compared to those with obesity.

2. Materials and Methods

This is a cross-sectional case-control study approved by the Research Ethical Committee of Hospital Universitário Pedro Ernesto (CAAE: 0190.0.228.000-10). All subjects signed the written informed consent before taking part in the study. This study was registered on Clinical Trials as "Study about the high-fat meal and postprandial lipemia" and numbered as NCT01692327.

2.1. Study Design and Participants. Women with obesity were selected from the Outpatient Care Unit for Obesity of the State University of Rio de Janeiro, Brazil. The subjects were divided into two groups: women with obesity (OBG) and controls (CG). We investigated the effects of diet intervention on microvascular reactivity. Before inclusion, participants underwent an assessment visit, which consisted of clinical, biochemical, anthropometric, and body composition evaluations and hemogram. Nineteen women with obesity composed the OBG while 18 lean women were allocated to CG. The main inclusion criteria were women, aged between 19 and 40 years and body mass index (BMI) between 30 and 34.9 kg/m^2 (OBG) and between 20 and 24.9 kg/m^2 (CG). Exclusion criteria were as follows: concomitant nutritional or pharmacological interventions; hypothyroidism, metabolic syndrome, any degree of glucose intolerance or diabetes mellitus; arterial hypertension; symptoms or a past history of lactose intolerance; weight gain or reduction by at least 5% of body weight in the six months preceding recruitment; tobacco use, alcohol abuse, and regularly practiced physical activities.

The biochemical analysis comprised the determination of blood levels of insulin, total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDL-c), and thyroid stimulating hormone (TSH) after a 12-hour overnight fast. Fasting and 2-hour after load (75 g, oral) glycemia were also evaluated. Glucose intolerance and metabolic syndrome were defined according to the American Diabetes Association (ADA) criteria [19] and the Joint Interim Statement [20], respectively.

Figure 1 schematizes the periods of the test. On this morning, after a 12 h fast, systolic (SBP) and diastolic (DBP), blood pressures were checked using the standard auscultatory method, and an intravenous catheter was inserted and maintained *in situ* throughout the test for blood samples withdrawal. Time points for blood pressure, microvascular reactivity assessment, and blood samples were as follows: fasting state (baseline) and 30, 60, 120, and 180-min after ingestion of a high-fat meal. Both groups received the same test meal. Participants had been instructed not to ingest fat-rich meals or to practice any physical activity within 24 hours before the test.

2.2. Test Meal (High-Fat Meal). Participants consumed a high-fat meal on the breakfast of the test day, composed of whole milk (200ml), chocolate (10g), margarine (20g), *croissant* (1 unit), cheddar cheese (60g), and salami (31 g). The meal was partially based on Signori and coworkers [21], with some modifications to include more palatable foods [22]. The meal consisted of 691.5 kcal and specifically, 24.8% of carbohydrates, 59.5% of lipids (being 21.9 g of saturated fat), and 15.7% of proteins. The time limit for ingestion of the meal was 10 minutes.

2.3. Blood Sample Analysis. Plasma levels of glucose were determined by glucose oxidase colorimetric method. Serum concentrations of TG, TC, and HDL-c were assessed by glycerol phosphate oxidase/peroxidase, cholesterol oxidase/peroxidase, and direct colorimetric methods, respectively. All analysis were performed by commercially available kits appropriated for the Automatic Analyser A25 (BioSystems, Barcelona, Spain), following the protocols provided by the kits manufacturer (BioSystems, Barcelona, Spain). LDL-c was calculated by Friedewald equation [23]. Intra- and interassay coefficient of variation of all analyses described above were below 15% and previously validated [24].

2.4. Microvascular Reactivity Assessment. Nailfold videocapillaroscopy (NVC) was performed and analyzed, by the same technician who was blinded to patient's data, according to our standardized, well-validated methodology, described elsewhere in the literature [24]. Images were recorded continuously (final magnification of x680) for later assessment of microvascular variables using Cap Image v7.2 software (Zeintl, Heidelberg, Germany).

Red blood cell velocity (RBCV) at rest, maximal red blood cell velocity ($RBCV_{max}$), and time taken to reach it ($TRBCV_{max}$) were evaluated. $RBCV_{max}$ was measured during post-occlusive reactive hyperemia (PORH) after 1-min ischemia, obtained by a pressure cuff (1 cm wide) placed around the proximal phalanx of the 4th finger and connected to a mercury manometer. We measured basal RBCV three times, and intra-assay coefficient of variation for all measurements ranged from 16.9 to 17.1%. During PORH, each variable was tested once. NVC was repeated on

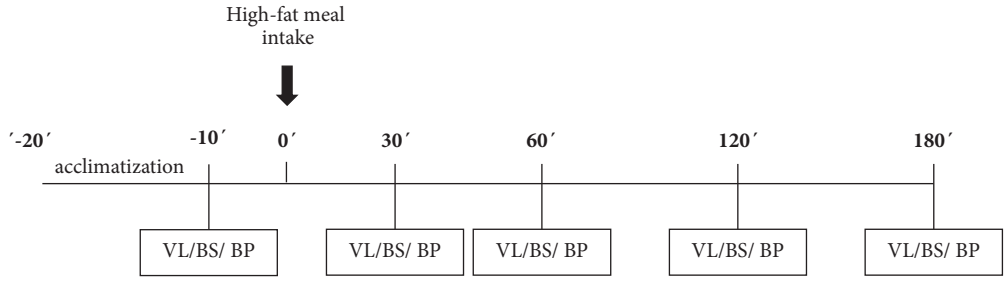

Figure 1: Experimental design. VL: videocapillaroscopy; BS: blood sample collection; BP: blood pressure.

nine subjects on different days, and the obtained inter-assay coefficient of variation ranged from 12.3% to 17.3%.

2.5. Statistical Analysis. Statistical analysis was performed by GraphPad® Prism software, version 5. Normal Gaussian distribution was assessed using the Shapiro-Wilk normality test. For Gaussian and non-Gaussian distributions, data were, respectively, expressed by mean±SD and median [1st-3rdquartiles]. ANOVA repeated measures or Friedman test performed intragroup comparisons. The unpaired *t*-test or the Mann-Whitney *U*-test compared variables between groups. We also proceeded individual regression equations for modeling the relationship between dependent variables (collected at fasting and during postprandial periods) and the independent variable (time after meal intake) which resulted in two variables for each subject that define the linear relationship between the dependent and independent variables, named as slopes and intercepts. The first one represents the steepness of the regression line. The higher the magnitude of the slope, the higher the rate of change. The intercept is the point where this regression line crosses the axis of the dependent variable. After the acquisition of slopes and intercepts, their mean values were used in the analysis. The statistical power for two-tailed comparisons between two independent groups was 0.95 with a α error probability of 0.05. $TRBCV_{max}$ (mean±SD) for group 1 and 2 were 11±1.73 and 9±1.35 seconds [25], respectively, estimating a sample size of 17 patients/group and a total sample size of 34 patients. GPower 3.1.10 software was used for power analysis and sample size estimation. A P-value <0.05 was considered significant.

3. Results

3.1. Clinical, Laboratory, and Body Mass Evaluation. The clinical, laboratory, and body mass characteristics of the study participants are depicted in Table 1. As expected, OBG had significantly greater weight (P<0.001), BMI (P<0.001), waist and hip circumferences (P<0.001), waist-to-hip ratio (WHR, P<0.001), DBP (P<0.01), and fat mass (P<0.001) compared to CG. Additionally, OBG had significantly higher levels of glucose (P<0.05), insulin (P<0.01), TC (P<0.05), LDL-c (P<0.01), and TG (P<0.05) in comparison to CG. On the other hand, OBG had significantly lower muscle mass when compared to CG (P<0.001). No significant differences between groups were found concerning age (P=0.083), height (P=0.73), heart rate (P=0.90), and HDL-c (P=0.33).

Table 1: Clinical, laboratorial, and body composition characteristics of control group (CG) and obesity group (OBG).

Variables	CG	OBG
Age (y)	27.89 (5.38)	30.74 (4.48)
Weight (kg)	57.82 (6.24)	86.28 (7.84)∗∗∗
Height (m)	1.63 (0.07)	1.63 (0.05)
BMI (kg/m^2)	21.81 (1.79)	32.31 (1.54)∗∗∗
WC (cm)	79.47 (5.09)	106.4 (9.37)∗∗∗
HC (cm)	100.3 (4.47)	117.5 (6.50)∗∗∗
WHR	0.79 (0.03)	0.91 (0.85)∗∗∗
SBP (mmHg)	109.1 (7.53)	115.8 (13.82)
DBP (mmHg)	69.06 (6.12)	76.74 (9.14)∗∗
Heart rate (bpm)	73.19 (9.56)	72.84 (6.40)
Fat mass (%)	27.88 (3.11)	38.12 (1.78)∗∗∗
Muscle mass (%)	72.14 (3.08)	61.81 (1.75)∗∗∗
Glucose (mg/dl)	81.06 (6.05)	88.79 (13.06)∗
Insulin (mU/l)	8.18 (4.25)	12.74 (4.76)∗∗
TC (mg/dl)	169.40 (32.18)	196.50 (29.24)∗
LDL-c (mg/dl)	86.70 (24.92)	110.40 (27.81)∗∗
TG (mg/dl)	73.72 (37.84)	109.50 (56.42)∗
HDL-c (mg/dl)	67.94 (17.65)	62.37 (16.57)

Data are expressed as mean (SD). BMI: body mass index; WC: waist circumference; HP: hip circumference; WHR: waist-to-hip ratio; SBP: systolic blood pressure; DBP: diastolic blood pressure; TC: total cholesterol; LDL-c: low-density lipoprotein cholesterol; TG: triglycerides; HDL-c: high-density lipoprotein cholesterol. ∗Significantly different compared to CG group; ∗P<0.05; ∗∗P<0.01; ∗∗∗P<0.001.

3.2. Plasma Glucose, Insulinemia, and Lipid Profile before and after the High-Fat Meal Intake. Figure 2 shows the differences between plasma glucose, insulinemia, and lipid profile between groups before and after the high-fat meal intake. In CG, after meal intake, glycemia increased at 30-min followed by a significant reduction at 60-min compared to baseline. Insulinemia also had a peak at 30-min but remained significantly elevated at 60 and 120-min compared to baseline. TC and HDL-c levels decreased significantly after a high-fat meal up to 180-min compared to baseline. LDL-c concentrations remained unchanged until 60-min and decreased significantly afterward when compared to baseline. TG did not change until 60-min, showing a significant progressive increment after that time point compared to baseline. In OBG, insulin and TG levels followed the same trend as in

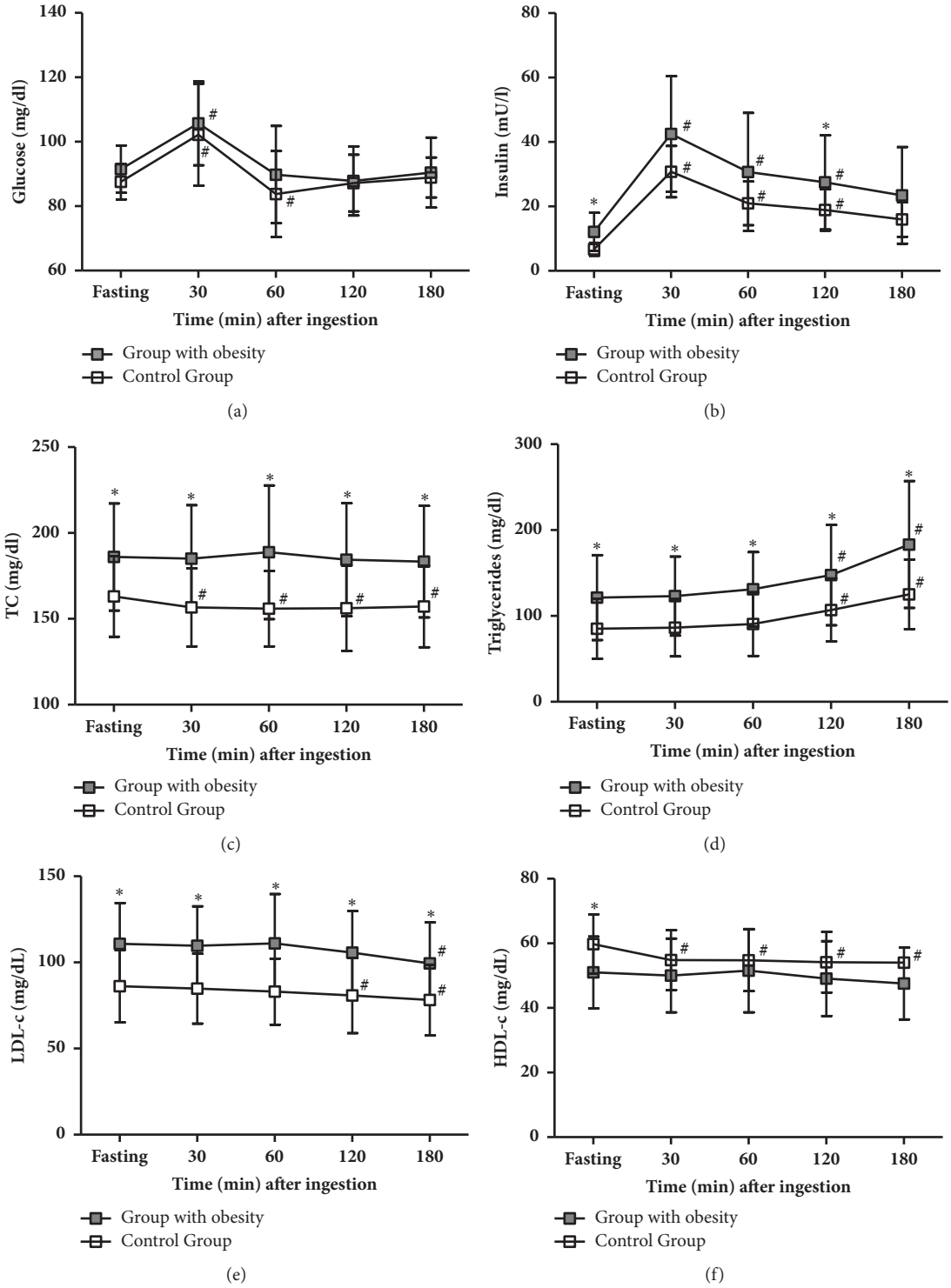

Figure 2: Metabolic responses to a high-fat meal in control group (CG) and with obesity group (OBG). TC: total cholesterol, LDL-c: low-density lipoprotein cholesterol, and HDL-c: high-density lipoprotein cholesterol.

CG. But in contrast, TC and HDL-c concentrations did not decrease after a high-fat meal in OBG. Differently from CG, in the OBG the levels of LDL-c remained unaltered until 120-min and became significantly reduced at 180-min compared to baseline. Additionally, plasma glucose levels increased at 30-min, despite a decrease at 60-min, no statistically significant decrease was observed in OBG.

Considering intergroup analysis, we noticed significant differences between groups in all-time points concerning TC, TG, and LDL-c. Serum levels of insulin in OBG were

significantly different from CG at rest and 120-min. In OBG, HDL-c was significantly different from CG at rest. No significant differences between groups were observed regarding plasma glucose levels. No difference in slopes^{-1} (data not shown) was noted for these variables while intercepts for insulin (18.5±4.34 versus 26.96±12.97, $P<0.05$), TC (159.5±22.66 versus 185.9±30.98, $P<0.01$), TG (80.70±34.16 versus 120.4±49.93, $P<0.01$), and LDL-c (86.0±20.78 versus 110.7±23.43 $P<0.01$) were higher in OBG compared to CG, respectively. No differences in intercept between CG and OBG were found regarding glucose and HDL-c.

In summary, a high-fat meal promoted a comparable two-phase response in both groups: one until 60-min, associated with increases in glucose and insulin, and another one, after 120-min, associated with an increase in triglycerides.

3.3. Microvascular Reactivity before and after the High-Fat Meal Intake. Microvascular reactivity differences between groups before and after the high-fat meal intake are shown in Table 2. In all-time points, OBG presented RBCV and $RBCV_{max}$ significantly lower when compared to CG whereas $TRBCV_{max}$ was significantly higher in comparison to CG ($P<0.05$), pointing to a microvascular dysfunction related to obesity.

RBCV were slower in the OBG at 30-min compared to baseline ($P<0.05$). $RBCV_{max}$ were significantly slower in CG at 30 min and 60-min and in OBG at 30-min in comparison to baseline ($P<0.05$). $TRBCV_{max}$ was significantly prolonged in CG at 30-min and 60-min and in OBG at 30-min when compared to baseline ($P<0.05$).

Although slopes^{-1} for microvascular variables did not add any new finding, intercepts revealed significant differences in microvascular reactivity between groups ($P<0.001$). In the intergroup comparison, OBG had microvascular dysfunction not only at baseline conditions, expressed by lower RBCV and $RBCV_{max}$ and prolonged $TRBCV_{max}$ compared to CG but also during all postprandial period (intercepts). These observed differences between groups throughout the postprandial period suggest a state of microvascular dysfunction in OBG throughout the tested period compared to CG. Of note, we emphasize that intragroup analysis showed that a high-fat meal elicited different responses between the groups: this diet did not influence RBCV in the CG whereas in OBG it was further reduced at 30-min. Additionally, variables tested during PORH ($RBCV_{max}$ and $TRBCV_{max}$) were influenced by a high-fat meal in both groups. While in CG these responses returned to baseline levels after 60-min, in OBG there was a more significant decrease and an earlier return to baseline levels (after 30-min).

In summary, OBG had microvascular dysfunction that was exacerbated by a high-fat meal while in the CG, an impairment of microvascular function was observed during the first 60-min of ingestion.

4. Discussion

To our knowledge this is the first study which demonstrates that high-fat meal elicits an impairment of microvascular function in lean women and aggravates microvascular dysfunction already present in women with obesity at fasting state (baseline conditions).

On this study, we initially demonstrated, as our main finding, that the high-fat meal aggravates microvascular reactivity during the postprandial period in both groups, although in those with obesity it does not recover until the end of the test (180-min). Additionally, during the first 60-min following meal intake we could notice the worst microvascular responses in both groups. Therefore, microvascular dysfunction in the OBG was present during fasting but deteriorated following a high-fat meal, although unexpectedly in lean controls a worse microvascular reactivity occurred after a high-fat meal intake during the first 60 minutes. The OBG participants were age and gender-matched to CG, and according to exclusion criteria they were nondiabetic, nonhypertensive, and they were not diagnosed with metabolic syndrome, suggesting that obesity per se was the primary cause for microvascular dysfunction [26, 27].

Previous studies performed by our group focused on fasting state [18, 24, 26, 27]. Therefore, we decided to investigate if microvascular alterations could be observed during the postprandial period after a high-fat meal since humans are frequently at non fasting state. Furthermore, subjects ingest large amounts of food, generally with high-fat content, consuming between 20 and 70 g of fat by meal [28]. This diet is associated with atherogenic lesions and is also considered an independent cardiovascular disease risk factor [29, 30]. In this study, we have demonstrated that during postprandial state, after a high-fat meal, there is an early functional involvement of the microvascular system, at the capillary level. It is possible to suppose that recurrent damaging bouts to the microvascular system following repeated ingestion of high-fat meals could have further deleterious effects to microcirculation.

During the postprandial state, reduced microvascular reactivity has been observed in obesity after a mixed meal, and this has been explained regarding impaired insulin sensitivity and postprandial hyperglycemia [31, 32]. These results are probably related to visceral adiposity, typically associated with metabolic and vascular complications. However, another study found that microvascular function was not reduced in healthy subjects after a mixed meal, suggesting that the physiological role of the microvascular system (delivery of nutrients and hormones to tissues) during meal ingestion was maintained during the postprandial period [33]. Our data contribute to the understanding of the microhemodynamic behavior of the microcirculation specifically at the capillary level, although we tested it after a high-fat meal. The originality of our data lies on the finding that a high-fat meal affected microvascular reactivity in both groups (controls and women with obesity)

Microvascular reactivity was tested in the fasting state, during the postprandial period, in resting conditions, and during the reactive hyperemia response as well. 1-min ischemia was performed to measure the effect of shear stress during the reactive hyperemic response. At the onset of reperfusion, there is a sharp rise in blood flow followed by

Table 2: Microvascular reactivity in the control group (CG) and with obesity group (OBG) at fasting and after high-fat meal intake.

Variables	Groups	Fasting	30′	60′	120′	180′	Slope^{-1}	Intercept
RBCV (mm/s)	CG	0.326[0.316-0.330]	0.3195[0.3117-0.3291]	0.3237[0.3148-0.3262]	0.3233[0.3113-0.3263]	0.3247[0.3194-0.3305]	2542[-11720, 13700]	0.9697[0.9548, 0.9884]
	OBG	0.3015[0.2866-0.3101]∗	0.2855[0.2723-0.2987]#∗	0.2910[0.2806-0.3053]∗	0.3047[0.2949-0.3195]∗	0.3043[0.2848-0.3167]∗	2043[-93490, 6253]	0.8697[0.8463, 0.8898]∗∗∗
$RBCV_{max}$ (mm/s)	CG	0.3607[0.3483-0.3640]	0.3510[0.3390-0.3577]#	0.3517[0.3390-0.3600]#	0.3583[0.3410-0.3633]	0.3627[0.3573-0.3677]	5604[-14440, 14090]	1.062[1.049, 1.081]
	OBG	0.3300[0.3173-0.3420]∗	0.3200[0.3012-0.3263]#∗	0.3240[0.3107-0.3305]∗	0.3293[0.3178-0.3418]∗	0.3270[0.3197-0.3340]∗	3973[-8382, 22145]	0.9606[0.9475, 0.9824]∗∗∗
$TRBCV_{max}$ (s)	CG	3.0[3.0-4.0]	4.5[4.0-6.25]#	4.0[4.0-5.0]#	4.0[3.0-5.0]	3.5[3.0-4.25]	-96.67[-316.4, 133.8]	3.733[3.267, 4.448]
	OBG	5.0[5.0-7.0]∗	7.0[6.0-8.0]#∗	7.0[5.0-8.25]∗	5.5[5.0-7.0]∗	6.0[5.0-7.0]∗	-61.05[-269.3, 114.9]	6.759[5.798, 7.259]∗∗∗

Data are expressed as median [1st-3rd quartiles]. RBCV: red blood cell velocity; $RBCV_{max}$: red blood cell velocity after 1min ischemia; $TRBCV_{max}$: time taken to reach RBCVmax. ∗Significant difference between groups; ∗P<0.05; ∗∗∗P<0.001. # Significant difference within group.

a gradual return to its baseline levels, influenced by accumulation of vasodilator metabolites and formation of ROS, customarily washed out, or destroyed by the bloodstream and smooth muscle cell reactivity. During reperfusion, the myogenic response, due to a rapid stretch of microvascular smooth muscle cells, is responsible for the return of blood flow to its baseline values. Microvascular reactivity during reactive hyperemia was associated with glucose metabolism [27] in the fasting state. We have noted that, after a high-fat meal in controls and especially in women with obesity, blood microflow did not return to its baseline values and was further worsened. Hypertriglyceridemia could act negatively on microvascular reactivity inducing a dysfunctional response during the postprandial period, particularly after high-fat meals. However, during our test, triglyceride levels increased only after 120-min while the worst microvascular reactivity was observed during the first 60-min of the test. During this period, insulin and glucose had increased levels among them, and only insulin levels were associated with microvascular reactivity. Therefore, we believe that hyperinsulinemia could better explain these disturbances of microvascular blood flow observed in both groups as a result of meal ingestion during the first hour of the test. Our study cannot exclude the influence of hyperglycemia and also of nonassessed biomarkers (like chylomicrons).

A remarkable point herein observed is the significant declining of microvascular hemodynamics in CG at 30 and 60-min that coincided with a significant reduction of HDL-c serum levels. HDL-c has antioxidant and anti-inflammatory properties, such as decrease of interleukin-1 induced expression of adhesion molecules, reduction of interleukin-8 (the chemotactic factor for neutrophils) expression, and the presence of high content of antioxidant enzymes, such as paraoxonase and glutathione peroxidase, in its structure [34]. Thus, we believe that the decreased HDL-c concentrations contributed to ischemia/reperfusion injury and therefore for the impaired microvascular function at these time points.

Some limitations warrant mention. Ideally, the observation period of the study should be extended to 360-minutes. With this more extended period of observation, the impact of postprandial lipemia would be possibly more apparent not only in blood samples but also on microvascular reactivity.

Our results are restricted to gender since we performed them only in women at menacme. Ketel and coworkers [35] found no differences in microvascular reactivity between phases of the menstrual cycle, although some researchers have suggested this. In this study, we did not perform the tests in the same phase of the menstrual cycle. Only women were recruited for the study because most of the individuals attended at Outpatient Care Unit for Obesity, at our University, were women. Moreover, our experience revealed that women attend frequently the consultations and exams and present greater adherence to clinical trials. In this study, the age range assigned as an inclusion criterion was 19 to 40 years to ensure that women were at menacme and, thus, to avoid menopause and its consequent negative impact on endothelial function due to estrogen deficiency [36], which could be a confounding factor for our analysis.

5. Conclusions

A high-fat meal worsened an established impairment of microvascular reactivity in women with obesity but without hypertension, diabetes, and metabolic syndrome and induced it in lean controls. This effect was more pronounced and prolonged in women with obesity, and, in these women, postprandial microvascular dysfunction was associated with higher levels of insulin following high-fat meal ingestion. Our study cannot elucidate the precise mechanism underlying our findings. A possible explanation may be that high-fat meal intake induces hyperinsulinemia, endothelial activation, and inflammation that acutely impair microvascular function in subjects with obesity tested in this study. Our results reinforce the association between high-fat meal intake and atherosclerosis, even in healthy lean subjects, and emphasize the importance of studying microvascular and metabolic changes in the postprandial period.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

Behavior of Smooth Muscle Cells under Hypoxic Conditions: Possible Implications on the Varicose Vein Endothelium

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Varicose veins are a disease with high incidence and prevalence. In the venous wall, the smooth muscle cells (SMCs) act in the vascular homeostasis that secretes multiple substances in response to stimuli. Any alteration of these cells can modify the function and structure of the other venous layers such as the endothelium, resulting in increases in endothelial permeability and release of substances. Therefore, knowing the cellular and molecular mechanisms of varicose veins is imperative. The aims of this study are to understand how SMCs of patients with varicose veins subjected to saphenectomy of the great saphenous vein react under hypoxic cell conditions and to determine the role of vascular endothelial growth factor (VEGF) in this process. We obtained SMCs from human saphenous vein segments from patients with varicose veins (n=10) and from organ donors (n=6) undergoing surgery. Once expanded, the cells were subjected to hypoxic conditions in specific chambers, and expansion was examined through analyzing morphology and the expression of α-actin. Further gene expression studies of HIF-1α, EGLN3, VEGF, TGF-β1, eNOS, and Tie-2 were performed using RT-qPCR. This study reveals the reaction of venous cells to sustained hypoxia. As significant differential gene expression was observed, we were able to determine how venous cells are sensitive to hypoxia. We hypothesize that venous insufficiency leads to cellular hypoxia with homeostatic imbalance. VEGF plays a differential role that can be related to the cellular quiescence markers in varicose veins, which are possible therapeutic targets. Our results show how SMCs are sensitive to hypoxia with a different gene expression. Therefore, we can assume that the condition of venous insufficiency leads to a situation of sustained cellular hypoxia. This situation may explain the cellular response that occurs in the venous wall as a compensatory mechanism.

1. Introduction

Chronic venous disease refers to morphological and functional anomalies of the venous system and includes a series of clinical manifestations of varying severity of which varicose veins (VV) are the most common [1, 2]. Within this pathology, family history, aging, hormones, obesity, and pregnancy are the most important risk factors [3–5]. The different epidemiological studies carried out worldwide have made evident that chronic venous disease is greatly variable in

its incidence and prevalence. According to the Framingham study, the incidence of varicose veins per year is 2.6 % in women and 1.9 % in men [6]. In Western countries, varicose veins can affect up to 80 % of the adult population [7].

In the venous wall, the smooth muscle cells (SMCs) have an important role in the reception and control of the signaling in venous wall [8–10]. Under normal conditions in vivo, vascular cells maintain a very low replication level and lack specialized structures [11]. However, as it is able to activate and respond to numerous inflammatory, immune and thrombotic stimuli to maintain integrity, it influences the endothelium. The endothelium can perform complex functions and is vital for the maintenance of vascular wall homeostasis [12]. Endothelial cells can secrete multiple substances in response to different stimuli [13]. Any alteration of these cells can modify the function and structure of the other venous layers, resulting in the appearance of phenomena such as thrombosis, increased endothelial permeability with edema, and toxic substance release, which can lead to inflammation, ischemia, and even cell necrosis [10, 14].

Numerous authors have revealed the cellular and molecular mechanisms of chronic venous disease [10, 15–17]. Shoab et al. [18] showed that the synthesis of vascular endothelial growth factor (VEGF) is imbalanced in patients with VV, revealing its importance in the disease. VV produces distension of the venous wall and loss of normal fluid shear stress, which can lead to cellular hypoxia [14, 19].

The aims of this study are to understand how the smooth muscle cells (SMCs) of patients with varicose veins subjected to saphenectomy of the great saphenous vein react in situations of cellular hypoxia and to determine the role of VEGF in this process. We examined hypoxia, inflammation, and quiescence markers such as hypoxia-inducible factor 1 Alfa (HIF-1α), egl nine homolog 3 (EGLN3), VEGF, transforming growth factor beta 1 (TGF-β1), endothelial nitric oxide synthase (eNOS), and TEK receptor tyrosine kinase (Tie-2) to address these aims.

2. Patients and Methods

2.1. Patients. Saphenous vein segments were obtained during surgery from organ donors (controls, n=6) and subjects with venous insufficiency (varicose veins, n=10). Informed consent to participate in this study was obtained from all of the subjects. The project was approved by the Clinical Research Ethics Committee of the Central University Hospital of Defense-UAH (37/17). The specimens were first visually inspected to check for the presence of damaged areas in the vein wall. The mean of the study population was control=44,80 ± 0,86 years of age and varicose veins=47,12±1,26 years of age.

The segments of saphenous vein in the control group were obtained from organ donors, with no history of venous insufficiency or proven reflux during organ extraction surgery. Segments of saphenous vein in the second group were obtained at the time of extraction from patients with primary venous insufficiency and clinically confirmed. All varicose veins used in the study were classified as type 2 according to CEAP classification (C2).

The specimens were placed in sterile culture medium (MEM; minimal essential medium) with 1% antibiotic/antimycotic (broth from Thermo Fisher Scientific, Waltham, MA, USA) and stored at 4°C for their transfer to the laboratory, where they were divided into two fragments, one fragment was processed to obtain smooth muscle cells from explants and light microscopy (immunohistochemistry), and the other fragment was used for molecular biology studies.

2.2. Cells Isolation and Culture. Under sterile conditions in a Class II laminar flow cabinet (Telstar AV30/70; Telstar SA, Madrid, Spain), segments of human vein were flushed several times with MEM under sterile conditions and then longitudinally cut open. After removal of the endothelial and adventitial layers by scraping, the medial layer was cut into small explants (1 mm2). Subsequently they were subjected to digestion in a 0,1% type I collagenase solution (Worthington) in MEM (1h a 37°C) shaking in a bath. The enzyme reaction was stopped by adding the same volume of culture medium then centrifuged at 200 g for 7 min and discarded the medium. These explants were placed on the culture surface of 25 cm2 in a Roux flask (Nyclon-Intermed; Nunc A/S, Roskildo, Denmark) to which 0,5 ml Amniomax complete medium (Gibco BRL, Life Technologies Carlsbad, CA, USA) had been added to maintain the humidity of the culture surface and to improve the adherence of explants. The culture flasks were then incubated in a vertical position at 37°C in the presence of 5% CO2 in a cultured oven for 2 h. Next, 2,5 ml Amniomax medium was added per flask, and the flasks incubated horizontally under the previous conditions. Care was taken to avoid movements that might cause the explants to become unstuck. The culture medium was carefully replaced twice a week.

Once the cells had grown to confluence, SMCs were subcultured by enzyme treatment. This involved withdrawing the medium and rinsing three times in 2 ml of Hank's balanced salt solution (Gibco BRL, Life Technologies), followed by the addition of 2 ml trypsin-ethylenediaminetetraacetic acid solution at 1:250 (Gibco BRL, Life Technologies) and incubation at 37°C for 5 min. The enzyme reaction was stopped by the addition of 4 ml of culture medium. The resultant cell suspension was centrifuged at 200 g for 7 min and the cell pellet was resuspended in 9 ml of Amniomax medium. These cells in suspension were once again placed in culture at a density of 3 ml per 25 cm2 Roux flask until a confluent monolayer was obtained in an incubator with humidified 5% CO2 atmosphere at 37°C.

After that, cells were trypsinized as above and they were seeded in 12 mm diameter round glass coverslips (Nunclon Delta Surface, Thermo Fischer Scientific; Roskilde, Denmark) at the number of 30.000 cells per coverslip, and they were maintained in the humidified incubator for 48 hours prior to being subjected to hypoxic conditions.

These conditions were to establish **4 study groups**: **Group I:** cells from healthy (CV-SMC) in normoxic conditions (NOR), **Group II:** varicose vein cells (VV-SMC) in normoxic conditions, **Group III:** CV-SMC in hypoxic conditions (HYP), and **Group IV:** VV-SMC in hypoxic conditions. The number of viable cells was determined by trypan blue

exclusion and counted in a Neubauer chamber. All experiments were performed in triplicate.

2.3. Hypoxia Studies. In parallel experiments under normoxic conditions at 48h growth both CV- and VV-SMC cells were subjected to hypoxia in a gas-generating pouch system with indicator (GasPack EZ Gas Generating Pouches; Becton Dickinson and Company, Franklin Lakes, NJ, USA) to reduce oxygen levels to ≤1% (according to the manufacturer) during 6 hours. Hypoxic condition was confirmed with the anaerobic indicator saturated with a methylene blue solution on each sachet. This solution turns from blue to colorless in the absence of oxygen (according to the manufacturer). After the hypoxic conditions, the cells continued growing in oxygenate culture medium during more than 50 hours.

2.4. Alpha-Actin Immunocytochemistry. Cells from this assay were used to determine the protein expression of the a-actin. Confluent SMCs were fixed in 4% paraformaldehyde for 10 min at 4°C. Once fixed, the cells were hydrated and equilibrated twice in PBS 1X (pH 7.4). Then, cells were permeated with PBS containing 0.1% Triton X-100, 1% BSA, and 10% FBS for 45 min at room temperature. After that, primary antibody anti α-actin (dilution 1:400) (Sigma-Aldrich) was applied overnight at 4°C. Cells were washed three times with PBS and incubated for 1 h at room temperature with the secondary antibody anti-mouse IgG-biotin conjugate (1:300) (Sigma-Aldrich) for α-actin detection. Then, samples were washed three times with PBS and incubated for 90 min at room temperature with ExtrAvidin-alkaline phosphatase (1:200) (Sigma-Aldrich) for α-actin detection. After washing with PBS, α-actin was revealed with Fast Red kit (Sigma-Aldrich). Nuclei were counterstained with light hematoxylin staining. After immunostaining, the cell cultures were examined under a light microscope (Zeiss).

2.5. Real Time RT-PCR. RNA was extracted through guanidine-phenol-chloroform isothiocyanate procedures using Trizol (Invitrogen, Carlsbad, CA, USA) from confluent smc cultures. The RNA was recovered from the aqueous phase and precipitated by adding isopropanol and incubating overnight at -20°C. RNA integrity was checked using a 1% (w/v) agarose gel and quantified by spectrophotometry. Complementary DNA was synthesized using 200 ng of the total RNA by reverse transcription with oligo dT primers (Amersham, Fairfield, CT, USA) and the enzyme MML V-RT (Invitrogen). The following specific cDNAs were them amplified by PCR (Table 1).

The RT-PCR mixture contained 5 μl of the inverse transcription product (cDNA) diluted 1:20, 10 μl of iQ SYBR Green Supermix (Bio-Rad Laboratories) and 1 μl (6 μM of each primer in a final reaction volume of 20 μl. RT-PCR was performed on a StepOne PlusTM System (Applied Biosystems-Life Technologies), using the relative standard curve method [20]. Samples were subjected to an initial stage of 10 min at 95°C. The conditions for cDNA amplification were 40 cycles of 95°C for 15s, 59°C (HIF-1α) or 60°C (EGLN3, TGF-β1, VEGF, eNOS, Tie-2 and GAPDH) for 30 s and 72°C for 1 min and a final stage of 15 s at 95°C, 1 min at 60°C, 15 s at 95°C and 15 s at 60°C. Fluorescence was determined at the end of each cycle. The data obtained from each gene are interpolated in a standard curve made by serial dilutions of a mixture of the study samples which is included in each plate. Gene expression was normalized against the expression recorded for the reference GAPDH gene. All tests were performed in triplicate. Results were expressed in Relative Quantity mRNA (RQ).

2.6. Statistical Analysis. For the statistical analysis, the GraphPad Prism® 5.1 program was used, applying the Mann–Whitney U test. The data are expressed as the mean ± deviation from the mean. The significance is set at $p < 0.05$ (∗), $p < 0.005$ (∗∗), and $p < 0.001$ (∗ ∗ ∗).

3. Results

3.1. The Effects of Hypoxia on Smooth Muscle Cells from Healthy (CV-SMCs) and Varicose (VV-SMCs) Veins. First, we investigated the behavior of smooth muscle cells from healthy and varicose veins during their expansion under *in vitro* conditions. We observed that, in oxygenated culture medium, CV-SMCs showed better adhesion and proliferation than those of VV-SMCs during the first eight hours (Figures 1(a) and 1(b)). The CV-SMCs also had higher protein expression levels of α-actin (Figure 1(b)). Subsequently, the CV-SMCs and VV-SMCs were subjected to experimental hypoxic conditions. Under these conditions, both cell populations were observed to have similar behavior (Figure 1(b)).

When studying cell behavior, differences were observed in the number of dead cells in both groups (Figure 1(c)). Under conditions of hypoxia (HYP), the CV-SMCs showed a significant increase in the percentage of dead cells with respect to the normoxic condition (NOR) (2.96±1.02 % NOR versus 9.38±0.97 % HYP, ∗∗$p<0.005$). A significant increase was also observed in the percentage of dead cells under hypoxic conditions for the VV-SMCs (9.44±1.08 % NOR versus 11.78±1.33 % HYP, ∗$p<0.05$). When comparing both of the study groups under normoxic conditions, the VV-SMCs showed a significant increase in the percentage of dead cells with respect to the CV-SMCs (∗∗$p<0.005$).

3.2. Expression of Hypoxia Markers. The expression of HIF-1α and EGLN3 was examined, and our results showed significant differences in the expression of these genes in the study groups under conditions of experimental normoxia and hypoxia (Figure 2).

For **HIF-1α,** the CV-SMCs under normoxic conditions were observed to have levels significantly higher than those under hypoxic conditions (93.17±8.42 RQ NOR versus 48.61±4.09 RQ HYP, ∗∗$p<0.005$). The VV-SMCs under normoxic conditions showed significantly higher levels than in the hypoxia group (136.59±8.72 RQ NOR versus 76.07±4.04 RQ HYP, ∗ ∗ ∗$p<0.001$). When comparing the CV-SMCs versus VV-SMCs groups, we observed significantly higher expression levels under normoxic condition (∗$p<0.05$) and

Table 1: RT-qPCR primer sequences and binding temperatures (Temp).

GENE	SEQUENCE Fwd ($5'\longrightarrow3'$)	SEQUENCE Rev ($5'\longrightarrow3'$)	Temp
HIF-1α	ACGTGTTATCTGTCGCTTTGAG	ATCGTCTGGCTGCTGTAATAATG	59°C
EGLN3	GATGCTGAAGAAAGGGC	CTGGCAAAGAGAGTATCTG	60°C
TGF-β1	GCGTGCTAATGGTGGAAAC	CGGAGCTCTTGATGTGTTGAAGA	60°C
VEGF	ATGACGAGGGCCTGGAGTGTG	CCTATGTGCTGGCCTTGGTGAG	60°C
eNOS	AAG AGG AAG GAG TCC AGT AAC ACA GA	ACG AGC AAA GGC GCA GAA	60°C
Tie-2	TGCCCAGATATTGGTGTCCT	CTCATAAAGCGTGGTATTCACGTA	60°C
GAPDH	ATGACGAGGGCCTGGAGTGTG	CCTATGTGCTGGCCTTGGTGAG	60°C

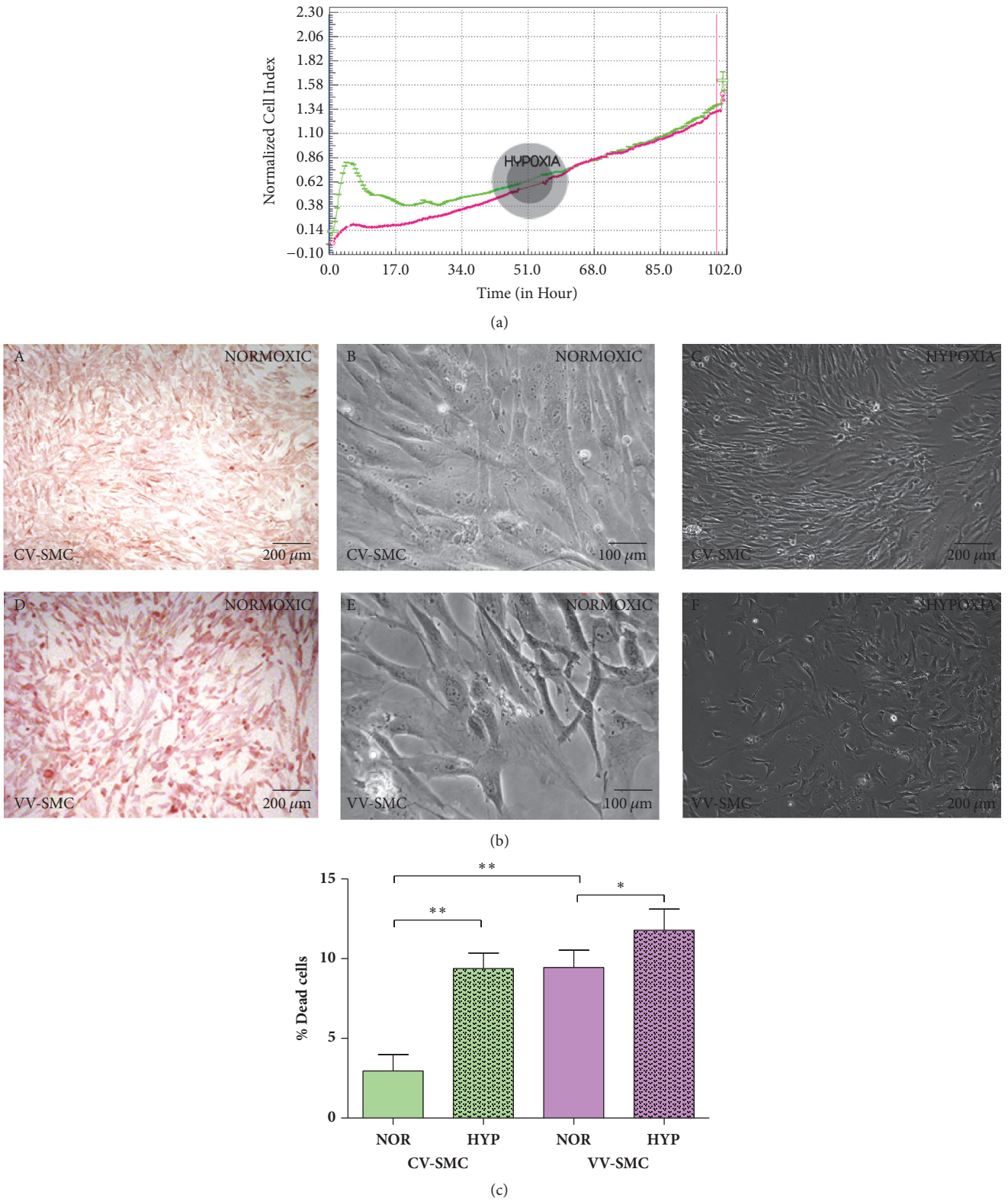

Figure 1: (a) Normalized cell index of cultures with control smooth muscle cells (CV-SMCs, green line) and varicose veins smooth muscle cells (VV-SMCs, purple line) veins under hypoxic conditions. (b) Images of CV-SMCs and VV-SMCs in which the differential expression of α-actin (A AND D) can be observed. Different morphology and proliferation were observed for the CV-SMCs and VV-SMCs under normoxic (B AND E) and hypoxic conditions (C AND F) via inverted microscopy. (c) Quantification of the percentage of dead cells in the CV-SMCs and VV-SMCs under normoxic (NOR) and hypoxic conditions (HYP). $p<0.05$ (*); $p<0.005$ (**).

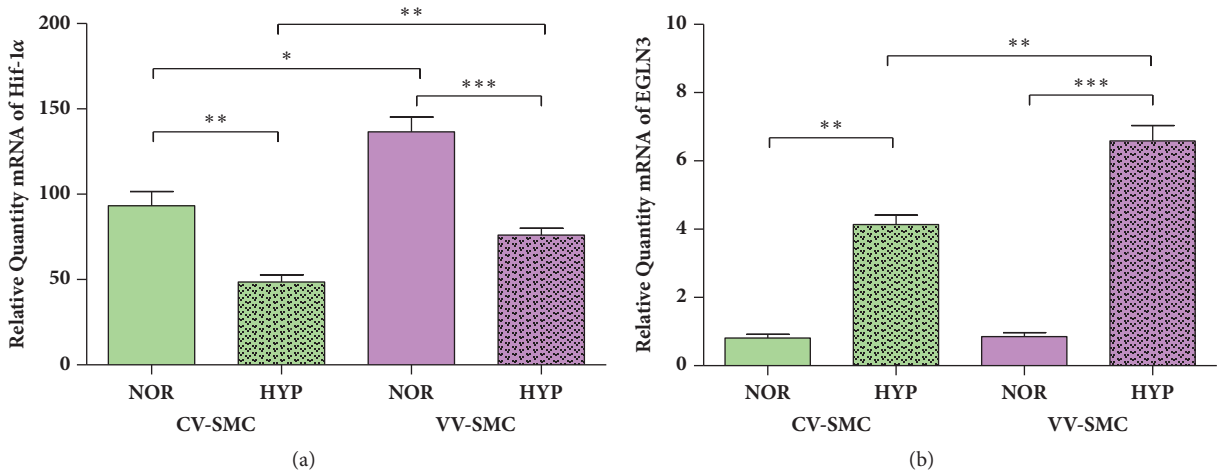

Figure 2: mRNA levels of HIF-1α (a) and EGLN3 (b) in smooth muscle cells from healthy (CV-SMCs) and varicose veins (VV-SMCs) patients under normoxic (NOR) and hypoxic (HYP) conditions. The results were normalized to that of the GAPDH gene and are provided in arbitrary units. The data are expressed as the mean ± standard deviation from the mean. The significance is set at $p<0.05$ (∗), $p<0.005$ (∗∗), or $p<0.001$ (∗ ∗ ∗).

experimental hypoxic conditions in VV-SMCs (∗∗$p<0.005$) (Figure 2(a)).

When studying the gene expression of **EGLN3,** a statistically significant elevation was found in the CV-SMCs under conditions of experimental hypoxia (0.81±0.11 RQ NOR versus 4.14±0.27 RQ HYP, ∗∗$p<0.005$). A similar trend was observed for the VV-SMCs during experimental hypoxia, though the levels were significantly higher than those in the CV-SMCs (0.86±0.12 RQ NOR versus 6.59±0.44 RQ HYP, ∗ ∗ ∗$p<0.001$). When comparing the study groups, statistically significant differences were established in the expression levels of EGLN3 under conditions of experimental hypoxia between the CV-SMCs and VV-SMCs (∗∗$p<0.005$) (Figure 2(b)).

3.3. Expression of Angiogenesis and Proliferation Markers. To analyze angiogenesis and proliferation, the gene expression of VEGF, TGF-β1, eNOS, and Tie-2 was studied using RT-qPCR under conditions of experimental normoxia and hypoxia *in vitro*. Our results showed that the expression of these markers is significantly different in the two study groups (Figure 3).

The expression of **VEGF** showed a significant increase in the CV-SMCs under conditions of experimental hypoxia (0.67±0.16 RQ NOR versus 8.58±0.59 RQ HYP, ∗∗$p<0.005$). In addition, in the VV-SMCs, VEGF showed a significant decrease under experimental hypoxia (4.21±0.23 RQ NOR versus 0.61±0.09 RQ HYP, ∗$p<0.05$). Under conditions of normoxia, the VV-SMCs were observed to have significantly higher expression levels of VEGF compared to the CV-SMCs (∗∗$p<0.005$). When comparing both study groups, the VV-SMCs showed a significant decrease in VEGF expression compared to that of the CV-SMCs under conditions of experimental hypoxia (∗∗$p<0.005$) (Figure 3(a)).

When **TGF-β1** was studied, the CV-SMCs were observed to have a significant increase in expression under conditions of experimental hypoxia (0.50±0.06 RQ NOR versus 1.14±0.15 RQ HYP, ∗∗$p<0.005$). For the VV-SMCs, no statistically significant differences were observed in the expression levels of TGF-β1 under conditions of experimental normoxia and hypoxia (0.81±0.08 RQ NOR versus 0.54±0.07 RQ HYP). When comparing both study groups, the VV-SMCs had significantly higher TGF-β1 expression levels than those of the CV-SMCs under normoxic conditions (∗∗$p<0.005$). Under experimental hypoxic conditions, these levels were significantly lower in the VV-SMCs (∗ ∗ ∗$p<0.001$) (Figure 3(b)).

The expression levels of **eNOS** were not significantly different in the CV-SMCs (0.09±0.02 RQ NOR versus 0.14±0.03 RQ HYP). However, when studying the VV-SMCs, a significant increase in eNOS expression was observed under conditions of experimental hypoxia (0.11±0.02 RQ NOR versus 0.36±0.04 RQ HYP, ∗ ∗ ∗$p<0.001$). Comparing both study groups, the expression levels of eNOS in the VV-SMCs were statistically significantly higher than those of the CV-SMCs under conditions of experimental hypoxia (∗∗$p<0.005$) (Figure 3(c)).

Finally, **Tie-2** was studied, and a significant decrease in its expression levels in the CV-SMCs was observed under conditions of experimental hypoxia (7.57±0.49 RQ NOR versus 5.87±0.59 RQ HYP, ∗∗$p<0.005$). In the VV-SMCs, a significant decrease in the Tie-2 expression levels was also observed with experimental hypoxia (3.40±0.25 RQ NOR versus 2.53±0.14 RQ HYP, ∗$p<0.05$). Furthermore, in conditions of normoxia, the VV-SMCs were found to have to significantly less Tie-2 than the CV-SMCs (∗∗$p<0.005$) (Figure 3(d)).

4. Discussion

Of the various noxae that can affect the venous wall and its function, as shown in previous studies, homeostatic balance and the ability to react to different conditions contribute to

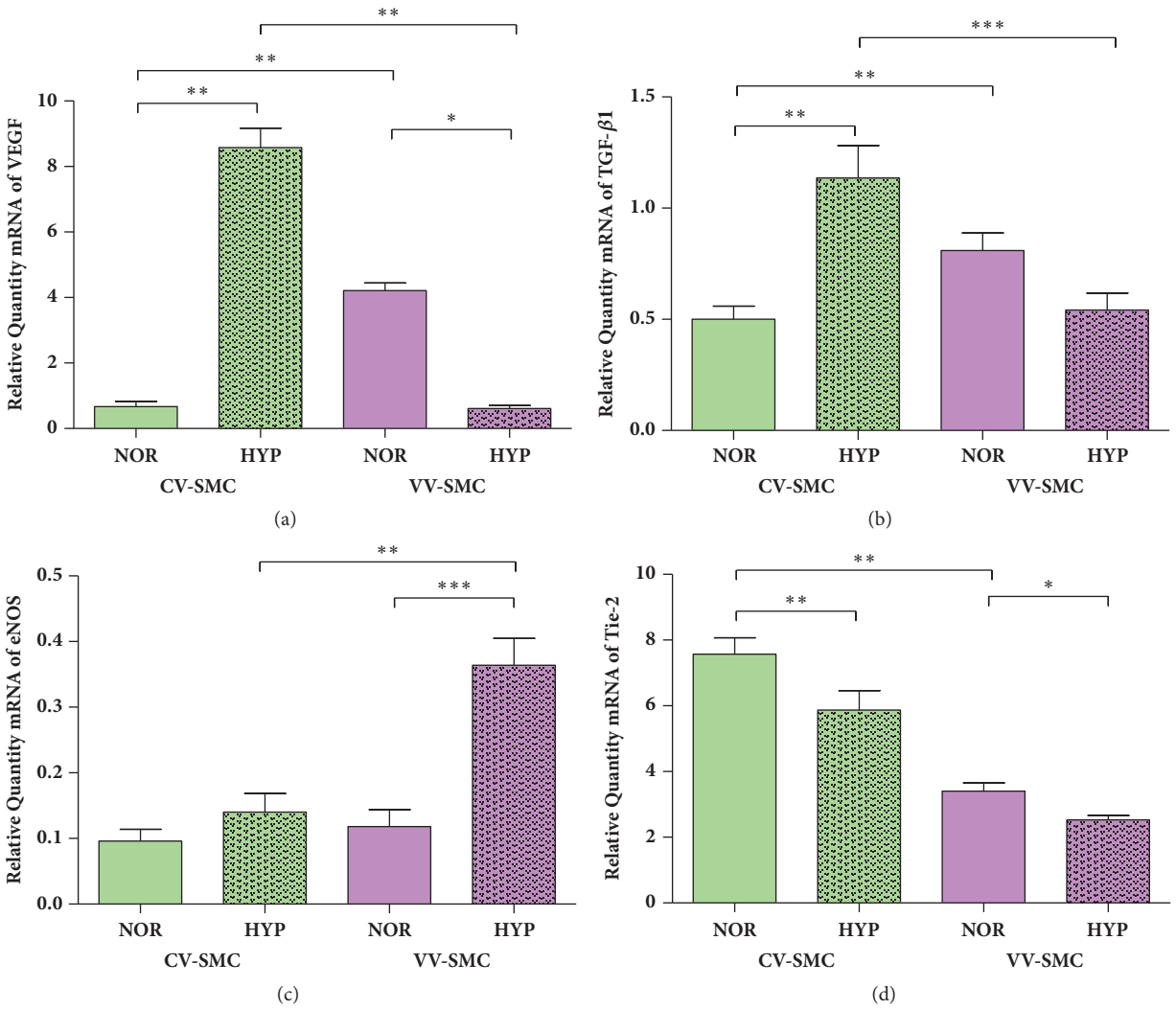

Figure 3: mRNA levels of VEGF (a), TGF-β1 (b), eNOS (c), and Tie-2 (d) in smooth muscle cells from healthy (CV-SMCs) and varicose veins (VV-SMCs) patients under normoxic (NOR) and hypoxic (HYP) conditions. The results were normalized to that of the GAPDH gene and are provided in arbitrary units. The data are expressed as the mean± standard deviation from the mean. The significance is set at $p<0.05$ (∗), $p<0.005$ (∗∗), or $p<0.001$ (∗ ∗ ∗).

venous failure [15, 16, 21, 22]. Venous insufficiency (varicose veins) produces venous wall dilatation, which leads to alterations in the structure of the compensatory wall, first in the form of areas of hypertrophy that, subsequent to failure, will become fibrosclerotic at the end of the process [13]. These induced alterations, encouraged and maintained by ischemic phenomena, lead to cellular activation, in turn causing repercussions at the functional level of the venous wall.

The ischemic process plays an important role in the process of venous wall insufficiency [23, 24]. Cellular behavior allows us to infer that microvascular dysfunction is the main alteration that should be considered [21]. The important role of VEGF in vascular pathology has been highlighted by numerous authors, with studies mentioning its role in angiogenesis and cell signaling [25, 26]. Bjarath et al. [27] noted that VEGF can cause remodeling of the venous wall in patients with varicose veins and that it could be associated with a genetic component. VEGF has been shown to play a role in the cellular response in different tissues in patients with venous insufficiency, conditioning the activity and response of affected tissue, such as the skin, in reepithelization processes in venous ulcers [25, 28]. Our results show that, in the controls, the expression of VEGF is significantly higher under hypoxic conditions compared to normoxic conditions. However, importantly, the levels of VEGF are significantly higher in SMCs from patients with varicose veins during normoxia, and they decrease under conditions of long-term hypoxia. These findings suggest that cells from patients with varicose veins suffering from hypoxia could be in a state of quiescence and could have significantly higher markers such as Tie-2. Some authors have reported that Tie-2 plays a role in the process of quiescence in vascular pathology, whereas in homeostasis, Tie-2 is a potential

angiogenic factor [29]. VEGF can affect the permeability of the venous wall and directly influence eNOS levels, which could explain the increased expression of this molecule under conditions of hypoxia and venous insufficiency [30].

Inflammation is another process factor that has been indicated to be essential for the progression of varicose veins [31]. Jin et al. [32] showed a strong relationship between the actions of VEGF and TGF-β1 in the pathophysiological processes that give rise to tissue damage. Moreover, VEGF and TGF-β1 have been shown to have different levels of gene expression depending on the conditions to which the cells are exposed [33]. Our results showed similar relative expression profiles for VEGF and TGF-β1; however, in absolute terms, the expression levels of VEGF were higher than all of the other studied markers.

Our results show that HIF-1α is differentially expressed in the SMCs from the study patients and that its expression is inversely related to the expression of EGLN3. Numerous authors have postulated that this pathway for hypoxia is deregulated in chronic venous disease, which causes the deregulation of the expression of these angiogenic factors [34–38]. In relation to this fact, EGLN3 has been shown to play an important role as an adsorber to hypoxic tissue and is also important for homeostasis [39]. Lee et al. [34, 35] described a significant imbalance of HIF-1α in the muscle layers of diseased vessels.

Knowing the cellular behavior of venous wall cells under conditions of normoxia and hypoxia is fundamental for understanding the venous wall as well as for identifying possible therapeutic targets that should be studied. The present study, despite having a preliminary character, should be considered that the cells are homogenized in the same culture conditions. The detection of significant differences between the studied populations acquires a great relevance for subsequent *in vivo* studies. Therefore, we can assume that the condition of venous insufficiency leads to a situation of sustained cellular hypoxia. This situation may explain the cellular response that occurs in the venous wall as a compensatory mechanism. In this study, we demonstrated two important facts. First, the muscle cells of people with varicose veins show levels of the markers studied similar to normal cells subjected to hypoxia (Hif-1α, VEGF, TGF-β1, and eNOS). That is, these cells have stable genetic or epigenetic changes, which are maintained after their stabilization in the culture medium. Secondly, we show that if this hypoxia continues in the long term, these cells no longer have the capacity to react and there is a failure of the factors that try to compensate the hypoxia (EGLN3). This fact would allow us to establish a correlation between the presence of certain markers in the patient's serum and the ability to react to sustained hypoxia. At this point, the new microRNA technology could help establish such a correlation.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Miguel A. Ortega and Beatriz Romero contributed equality. Julia Buján and Natalio García-Honduvilla are sharing senior authorship.

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Review Article

Interactions between the Cyclooxygenase Metabolic Pathway and the Renin-Angiotensin-Aldosterone Systems: Their Effect on Cardiovascular Risk, from Theory to the Clinical Practice

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Coronary artery disease (CAD) and stroke are the most common and serious long-term complications of hypertension. Acetylsalicylic acid (ASA) significantly reduces their incidence and cardiovascular mortality. The RAAS activation plays an important role in pathogenesis of CVD, resulting in increased vascular resistance, proliferation of vascular-smooth-muscle-cells, and cardiac hypertrophy. Drugs acting on the renin-angiotensin-aldosterone system (RAAS) are demonstrated to reduce cardiovascular events in population with cardiovascular disease (CVD). The cyclooxygenase inhibitors limit the beneficial effect of RAAS-inhibitors, which in turn may be important in subjects with hypertension, CAD, and congestive heart failure. These observations apply to most of nonsteroidal anti-inflammatory drugs and ASA at high doses. Nevertheless, there is no strong evidence confirming presence of similar effects of cardioprotective ASA doses. The benefit of combined therapy with low-doses of ASA is—in some cases—significantly higher than that of monotherapy. So far, the significance of ASA in optimizing the pharmacotherapy remains not fully established. A better understanding of its influence on the particular CVD should contribute to more precise identification of patients in whom benefits of ASA outweigh the complication risk. This brief review summarizes the data regarding usefulness and safety of the ASA combination with drugs acting directly on the RAAS.

1. Introduction

Activation of the renin-angiotensin-aldosterone system (RAAS) plays an important role in the pathogenesis of cardiovascular disorders, resulting in increased vascular resistance, excessive fluid retention, increased proliferation of vascular-smooth-muscle-cells, and cardiac hypertrophy. Inhibition of the bradykinin degradation, catalysed by the angiotensin converting enzyme (ACE), results in increased prostacyclin release and constitutes one of the additional mechanisms of the ACE inhibitors (ACE-I) antihypertensive effect. Bradykinin, physiologically degraded by ACE, is accumulated resulting in vasodilatation, which is partially dependent on the prostanoid metabolic pathways, nitric oxide, and endothelium-derived hyperpolarizing factor (EDHF). The cyclooxygenase (COX) inhibitors affect the above-mentioned mechanisms and may limit some beneficial effects of ACE-I. This in turn might be important in patients with hypertension, coronary artery disease (CAD), and congestive heart failure (CHF). These observations apply to most of nonsteroidal anti-inflammatory drugs (NSAIDs) and aspirin (acetylsalicylic acid, ASA) at high doses. Nevertheless, there is no strong evidence, based on the long-term prospective studies, confirming the presence of similar effects of ASA when administered in small, cardioprotective doses [1].

2. Renin-Angiotensin-Aldosterone System and the Cyclooxygenase Metabolic Pathway

Recent studies have clearly demonstrated the role of aldosterone in the pathogenesis of systemic inflammation,

endothelial dysfunction, and fibrosis [2, 3]. It has also been shown that the effects of mineralocorticoids are present not only in the kidneys and that they are synthesized also outside the adrenal cortex [4]. Aldosterone acts on the cardiovascular system and also on the mechanisms independent of blood pressure regulation. It has been proven that the pharmacological blockade of its receptor exerts protective effects on the cardiovascular system, even while maintaining a constant value of blood pressure [5, 6].

A large amount of data indicates the participation of COX-dependent pathways in the pathogenesis of endothelial dysfunction induced by aldosterone. Its development is also possible in the normotension, and it is suppressed by both aldosterone receptor blockade and the COX inhibition [7]. Further analysis of the connections between these elements has shown that the thromboxane (TX) receptor blockade reduces the severity of endothelial dysfunction induced by aldosterone, but similar results were not observed for the inhibition of its synthesis. The increased production of TXA2 by vessels with aldosterone-induced endothelial dysfunction has also been shown, which—in combination with the previously cited observations—would indicate the TXA2 as a key mediator in development of aldosterone-mediated endothelial dysfunction. However, taking into account the fact that the thromboxane synthesis inhibitors do not inhibit the vascular reaction to acetylcholine, it is postulated that the TX does not have a significant pathogenic role, which does have other prostanoids acting through the TX receptor [8–11].

Prostacyclin (PGI), apart from the recognized vasodilatory action, can pave the pressor effect *via* the TX receptors, which takes place, for instance, in the aorta, where PGI seems to have, next to the PGH2, the largest share in the activity of EDCF [12, 13]. Moreover, it has been shown that in certain situations the inhibition of prostacyclin synthesis can improve the diastolic reactivity of the vessel.

According to Blanco-Rivero [7], exposure to aldosterone induces endothelial dysfunction *via* activation of COX-2 both in case of initially normal blood pressure as well as in experimental models of hypertension. In the second case, the PGI2 appears to play a key role, but under normotensive conditions it is assigned to different prostanoids (PGE2, PGF2).

Another interesting aspect of the mineralocorticoids activity is their different effect on the endothelium, dependent on the exposure duration. In the short term, a vasodilatory reaction is observed which is explained by activation of NO-dependent pathways, as well as an effect of vasodilatory acting prostanoids. However, a clear tendency to vasoconstriction, which can be explained by the development of inflammatory processes, is observed in the long-term exposure and it exemplifies the negative consequences of the chronic RAAS hyperactivity.

3. Endothelial Function

Endothelial dysfunction, present in almost all of the cardiovascular disorders (CVD), develops as a result of nitric oxide and other vasodilators' deficit, with superiority of strong vasoconstrictive factors of proinflammatory action, such as endothelin I, angiotensin II, or some of the prostanoids [26, 27].

A widely understood imbalance among the whole group of factors with pressor activity [TXA2, isoprostanoids, 20-Hydroxyeicosatetraenoic acid, superoxide anion, H_2O_2, endothelin-1, angiotensin II, and UTP] and vasodilators [adenosine, prostacyclin (PGI2) and nitrogen oxide (NO), epoxyeicosatrienoic acids (EETS), and C-natriuretic peptide] may play a potential role in vascular tone regulation. The cooperation of endothelial cells with vascular-wall-smooth-muscle-cells is possible through the gap-junctions. It is manifested, i.e., by conduction of electric potential in response to EDHF or by transfer of small ions [28].

The beneficial effect of ASA in CVD is often attributed to its antiaggregative properties. The influence of ASA and other NSAIDs on the pro- and anti-inflammatory activity of endothelium is almost completely ignored. Retention of sodium, exacerbated by COX, can be compensated by other mechanisms to prevent the blood pressure increase. On the other hand, prostaglandin E2 and prostacyclin I2 are the determinants of renin secretion. Interfering with their metabolism may contribute to development of hypertension, especially in the course of renal artery stenosis. Fujino et al. [29] have shown that the deficiency of prostacyclin (PGI2) receptor prevents the development of renovascular hypertension.

Common transduction system beginning from the second messenger (cAMP) for prostacyclin, PGE2, EP2, and EP4 receptors may suggest the occurrence of similar effects after functional blockade of the EP2 and EP4 signalling [30]. Nevertheless, the study by Fujino et al. does not confirm this hypothesis, indicating clearly the key role of PGI2 and its receptors in the development of renovascular hypertension. The exact mechanism of this interaction remains unknown. A lot of data indicates the possibility of modulating the local activity of the adrenergic system to be responsible for the renin release regulation from the juxtaglomerular apparatus. Another hypothesis, opposing the results of Fujino et al., assumes the existence of an interaction at the level of dense dot stimulating the release of renin in response to a reduced chloride content in the urine. Decreased expression of the second symporter Na, K/2Cl (2NKCC2), leads to activation of COX-2 and increased synthesis of PGE2 followed by EP2 receptor activation and in consequence by releasing a greater amount of renin [31–33].

To summarize, the paradoxical duality of the prostanoids' influence on the regulation of blood pressure should be noticed. On one hand, there is an activation of the RAAS by COX-2-mediated PGI2 and, on the other hand, vasodilatory effect of PGI2 on blood vessels is exerted by endothelium and intensification of natriuresis. Under physiological condition, the state of dynamic balance between these opposing mechanisms is determined, and it is significantly altered in pathology. Further understanding of these complex interactions should allow the selection of two different groups of patients: the first one where the use of NSAIDs exerts adverse hypertensive effects and the second one with significant benefits of pharmacological blockade of the cyclooxygenase activity.

4. Platelet Function

Platelets have a well-defined role in coronary artery thrombosis and in other cardiovascular disorders, including diabetes mellitus, stroke, and peripheral vascular disease.

ASA acts by irreversible acetylation of the COX1 serine residue which blocks the access of substrate, arachidonic acid, to the catalytic site of the enzyme limiting the thromboxane A2 synthesis leading to the inhibition of platelet aggregation [34–37]. Aspirin can also acetylate other proteins, like albumins or haemoglobin, but acetylation of the COX1 enzyme occurs at micromolar concentrations, which suggests the specificity of this reaction. The impact of other NSAIDs on platelet function also has been investigated. None of them irreversibly inhibited COX1 due to another mechanism of drug attachment to the enzyme. Some of the NSAIDs may interact with ASA during their concomitant administration. It has been found that ibuprofen and its derivatives prevent the access of aspirin molecule to the acetylation site and thus prevent the irreversible inactivation of this enzyme. This theory was confirmed in Catella-Lawson et al. study where the administration of ibuprofen before aspirin decreased the amount of inactivated platelets. This observation made at the molecular level has significant clinical implications because the aggregation occurs only in 85-90% of platelets. Theoretically, this interaction can be circumvented by administering ASA before ibuprofen, which was shown to be effective [36]. There were no similar interactions with the administration of ASA and rofecoxib (affinity for COX2) as well as diclofenac and acetaminophen (weak affinity for COX1) [36].

Angiotensin II contributes to increased platelet aggregation by activating the G protein-coupled receptor. This increases the Ca^{2+} concentration in platelets, which promotes fibrinogen receptor expression, increases thromboxane A2 production, and—in consequence—causes aggregation. ACE-I should thus inhibit platelet aggregation. Some studies confirm this by use of captopril ex vivo [38], but other authors obtained opposite results [39]. A study by Moser et al. [40], in which the impact of captopril, enalapril, and fosinopril on platelet functioning in hypertensive patients was investigated, showed only minimal impact of these drugs on the platelets aggregation, probably due to their antihypertensive action. However, the impact on thromboxane A2 formation varied depending on the substance used. Administration of captopril inhibits the formation of TXA2 but only for a short period of time. Enalapril does not affect its concentration, whereas fosinopril blocks the formation of thromboxane A2 probably through direct antagonist effect on TXA2 synthase activity. This may indicate the utility of fosinopril in limiting platelet activation.

Angiotensin receptor blockers, despite their ACE-I-like action, have additional properties affecting platelet function. One of them, irbesartan, reduces the concentration of TXA2 similarly to the above-mentioned fosinopril. The exact mechanism of this action is unknown, but it is suspected that it could act directly or by its metabolites on the TXA2 receptor [41]. Furthermore, losartan causes the release of nitric oxide from platelets and endothelial cells which activates the phosphorylation of TXA2/PGH2 receptors and inhibits intraplatelet TXA2 pathway [42].

Aldosterone antagonists also affect the function of platelets. Schafer et al. analyzed the impact of eplerenone on vascular function and platelet activation demonstrating reduced platelet aggregation following its administration in diabetic rats. This is accompanied by reduced production of superoxides and elevated nitric oxide bioavailability. Similar observations were done with spironolactone [43].

5. RAAS in Oxidative Stress, Aging, and Inflammation

Chronic oxidative stress is an important mediator in the development of hypertension. It has been shown that the superoxide dismutase (SOD) activating agents lead to the inhibition of endothelial apoptosis and may prevent its dysfunction [44, 45].

Pharmacological blocking of endothelin receptors in experimental models of diabetes leads to reduction of the NADPH oxidase expression and consequently significant reduction in oxidative stress [46, 47]. A similar effect has also been shown for drugs inhibiting the RAAS [48, 49].

Paradoxically, hypertension induced in animal models is characterized by increased activity of NO-mediated pathways in response to Ach stimulation, with relatively constant basic NO-release. It is considered as a compensatory mechanism, which is relatively rapidly downregulated [50, 51].

Aging also comes with a decrease in the EDHF expression, which results in reduced vasodilatory reactivity in NO-independent mechanisms [52, 53]. This effect may be partially reversed by the treatment with candesartan, most likely due to upregulation of the gap-junctions between endothelium and vascular-smooth-muscle-cells [54], and it is also influenced by the calcium channel blockers (CCBs), selective estrogenic receptor modulators (SERMs), or vasopeptidase inhibitors [55–57].

A study by Mukai et al. [58] evaluating effect of the RAAS pathway blockade on reducing the severity of age-related endothelial dysfunction associated in rats showed that long-term suppression of the RAAS leads to a significant reduction of endothelial dysfunction by reducing the excessive synthesis of COX2 products and superoxide anion.

Interesting data on chronopharmacological activity profile of acetylsalicylic acid is provided by Hermida et al. [59–62]. The results of most of them clearly indicate its hypotensive action when the drug was administered in the evening, which is not observed while taking it in the morning. The observed daily variability of ASA pharmacological profile can be explained by several mechanisms. One of them is the increased clearance of ASA after administration in the morning [63]. A potential mechanism that could explain this phenomenon is the effect of ASA on the thromboxane and prostacyclin synthesis, also presenting the circadian variations [64–66].

Donors of sulfhydryl groups (e.g., N-acetylcysteine), through the antioxidant properties, can potentiate the hypotensive effects of drugs via the nitric oxide dependent mechanisms, which is also observed in the case of ACE-I [67].

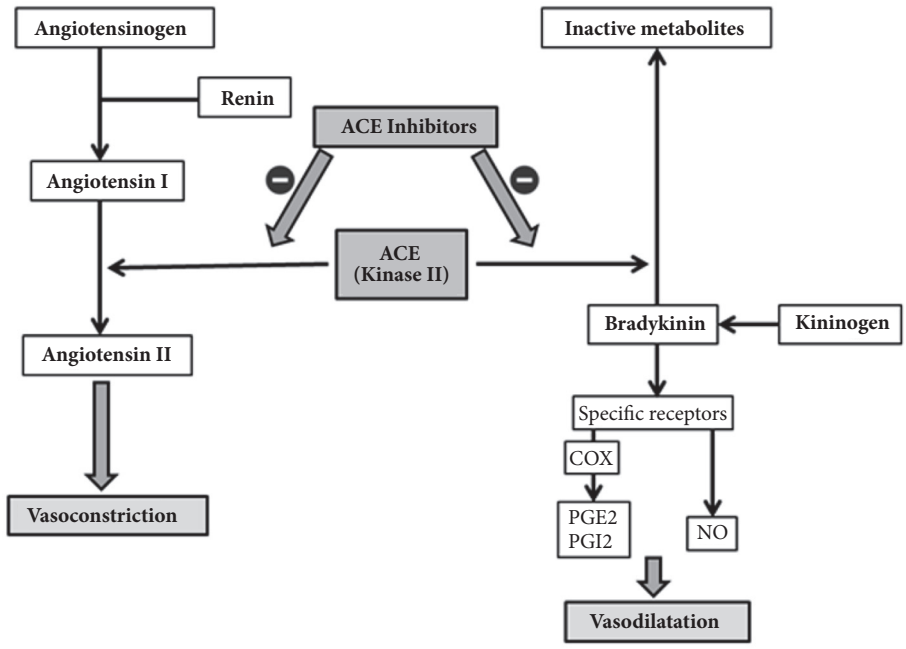

Figure 1: Theoretical background for an interaction between cyclooxygenase and the renin-angiotensin-aldosterone systems.

Endothelial and insulin signalling pathways have crosstalk between each other and therefore the relationship between endothelial function and insulin metabolism is very important. Insulin resistance impairs vascular reactivity and increases cardiovascular risk. Involvement of insulin resistance and endothelial dysfunction in pathological disorders contributes to impairment in the NO-dependent vasodilatation, cellular glucose uptake, enhancement in oxidative stress and inflammation (e.g., through increased synthesis of prostanoids and ROS as side products), hyperactivation of the RAAS, and increased generation of leading to atherosclerosis [68]. Regular aerobic physical activity seems to restore the homeostasis resulting in endothelial function recovery [69].

6. ASA and ACE-I: Their Pleiotropic Action

One clinical trial has shown the beneficial effect of aspirin on endothelial function and blood pressure control in hypertensive patients receiving statin. There were no similar results in a group without hypolipemic treatment, which may indicate the presence of synergism promoting pleiotropic effects of statins. Increase in the NO-bioavailability by ASA can contribute to the improvement of endothelial vasodilatory function and, as a result, a better control of blood pressure [70, 71]. On the other hand, inhibition of COX activity can reduce the beneficial effect of ACE inhibitors on endothelial vasodilatory function (Figure 1) [72].

In a study on diabetic patients with albuminuria it has been shown that addition of aspirin (at a dose of 300mg/24h) to enalapril does not abolish its nephroprotective properties because it does not lead to a reincrease of proteinuria previously reduced by the enalapril [73]. In one of the studies it has been shown that the interaction between aspirin and ACE inhibitors is observed at a dose of 500mg/24h ASA and does not occur when the daily dose of aspirin is 100 mg [20].

The results of some experimental studies have shown that the positive effect of ACE inhibitors on the postinfarction cardiac remodeling is mediated by bradykinin. Cardioprotective effect of ACE inhibitors by antioxidant activity is an effect of the activation of bradykinin receptor B2 and prostaglandin synthesis. This, in turn, could explain the reduction of this effect by COX inhibitors. Meune et al. demonstrated a dose-dependent negative effect of aspirin on the function of vascular wall in patients with heart failure treated with ACE inhibitor [74].

The blockade of cyclooxygenase may be particularly unfavorable in patients with heart failure treated with ACE-I, when limiting the prostanoid dependent activity of bradykinin causes an increase in peripheral resistance, a decrease in organ perfusion, and cardiac output. A different interaction profile is notable, depending on the selectivity of COX inhibitors—antagonism is greater with the increase of COX-1-selectivity. It may explain the clear pharmacodynamic antagonism observed between relatively more COX-1 selective indomethacin and ACE-I and in the case of ASA—a compound with comparable inhibitory activity on both isoforms of cyclooxygenase. The observed interactions are considerably poorer, especially for low ASA doses, when this affinity for tissue isoform of COX-1 is relatively smaller compared to the enzyme present in the platelets. Interestingly, in young healthy subjects, the presence of poor platelet responsiveness to ASA may be associated with development of endothelial dysfunction [75] and the common link could be the platelet carbonic anhydrase II [76].

Bhagat et al. showed that high doses of aspirin (i.e., 1000 mg/24h) inhibit the vasodilatation in response to

arachidonate, which was absent when small doses of ASA were applied. However, in a few other studies conducted on patients with congestive heart failure (CHF), it was demonstrated that 75-150mg of ASA is capable of inhibiting the arachidonate-dependent vasodilatation. This observation can be explained by the greater efficacy of ASA in inhibiting the synthesis of prostacyclin in this group of patients [77].

7. Clinical Evidence on Interaction of ASA with ACE-I, Angiotensin Receptor Blockers (ARB), and Mineralocorticoid-Receptor-Antagonists (MRA)

7.1. Coronary Artery Disease (CAD). Safety of the interaction between aspirin and ACE-I in the treatment of CAD has been the subject of numerous studies, but most of the data come from retrospective analyses. The GISSI-3 study [78] did not show the negative consequences of combining aspirin with ACE inhibitors (mortality in the placebo group and ACE-I without ASA, respectively, 13 and 10.8% vs. 6 and 5.4%, respectively, in the groups receiving aspirin concomitantly), and in the coexistence of diabetes combination of ASA and ACE-I led to a partial reduction of beneficial effects of ACE-I but still implied a significant reduction in mortality (the use of ACE-I reduced the mortality rate from 10.1 to 7.7% in the group previously treated with ASA and, respectively, from 24.7 to 13.5% in the group without ASA). The cohort study involving patients with CAD does not confirm the presence of pharmacodynamic antagonism between aspirin and ACE-I. Therefore the authors postulate the coadministration of these drugs in this group of patients. The results of the GISSI-3 show that concomitant use of aspirin and ACE-I is associated with a significant reduction in mortality in the acute phase of a heart attack. The CONSENSUS II study [14] demonstrated that the 6-month mortality in the patients with acute myocardial infarction and receiving concomitantly ACE inhibitors and aspirin is significantly higher, compared to patients treated with only one of these drugs. The CATS study (captopril and low-dose ASA) did not confirm this observation [15].

Administration of aspirin in combination with ARB in patients with CAD was examined by Levy et al. on the population from OPTIMIZE-HF study. The results showed no statistically significant side effects of coadministration of drugs from these two groups in a 60- and 90-day long period following discharge from the hospital [20]. In the case of coadministration of ASA and MRA (spironolactone and eplerenone), the addition of eplerenone to optimal medical therapy reduces morbidity and mortality among patients with acute myocardial infarction complicated by left ventricular dysfunction and heart failure. In the studied group, 89% of patients were given aspirin, as a part of optimal treatment [79, 80]. Additionally, the EPHESUS trial showed that, especially in patients with previous myocardial infarction, the early (3-7 days) addition of eplerenone to the optimal treatment significantly improved their prognosis [24, 81].

7.2. Congestive Heart Failure (CHF). There is also some evidence for the positive interaction between aspirin and ACE-I leading to inhibition of thromboxane synthesis (in the case of ACE-I by blocking the synthesis of ATII) in patients with CHF. Furthermore, bradykinin is a potential factor releasing norepinephrine in synapses of the sympathetic nervous system. In this case, the use of aspirin can possibly decrease the adverse consequences of sympathetic nervous system stimulation, as one of the mechanisms involved in noradrenaline release in the myocardial sympathetic system is activation pathway mediated by prostanoids. However, the results of the small study conducted on 40 patients with an acutely decompensated CHF do not confirm the existence of such a relationship—the addition of ASA to ACE-I resulted in the deterioration of hemodynamic parameters and abrogated the beneficial effects of ACE-I [25, 82–86]. A study by Pedone et al. did not reveal the existence of a negative interaction between aspirin and ACE-I in a group of elderly subjects with CHF. The results of the SOLVD study are in opposition to the ones referenced above [16, 87]. Considering the mechanism of ASA action, it has been postulated that the inhibition of prostacyclin synthesis by greater doses of aspirin reduces the beneficial effect of ACE-I. The SOLVD study demonstrated that the addition of aspirin in patients treated with ACE inhibitors reduces the clinical benefit while adding the ACE-I reduces the beneficial effect of aspirin on the survival. Data from the WASH (Warfarin/Aspirin in Heart Failure) and WATCH (Warfarin and Antiplatelet Therapy in Chronic Heart Failure) studies showed a clear increase in hospitalizations for CHF in the group randomized to receive aspirin, thus indicating no benefit of ASA adding to the CHF therapy [17].

The OPTIMIZE-HF study demonstrates the absence of side effects of combining aspirin with ARB in both ischemic and nonischemic CHF. Similar conclusions were postulated by Chan Su Min et al., who studied the population of the CHARM trial, where no negative effect of candesartan supply with ASA on mortality in patients with chronic heart failure was shown [18]. Coadministration of ASA and the aldosterone antagonists reduce the risk of death in both the ischemic and nonischemic CHF [76].

7.3. Arterial Hypertension (HTN). Another noteworthy issue is the significance of the aspirin and ACE-I interaction in the treatment of hypertension. Some meta-analyses indicate the possibility of an increase in blood pressure (RR) during the administration of selective COX-2 inhibitors. However, the observed changes tend to be too small to cause a significant increase in the relative risk [21], and in the case of ASA they are observed only for high doses [88]. In the study by Doroszko et al. it has been demonstrated that endothelial dysfunction in subjects with essential hypertension results not from decreased NO synthesis, but also from its increased degradation by reactive oxygen species (ROS) produced in the course of prostanoids' biosynthesis. Hence, limiting the activity of the arachidonic acid cascade could limit the impaired endothelial vasodilative reactivity in these patients [89].

Table 1: Data on the outcomes of combined use of ASA and ACE-I, ARB, and MRA in CAD, CHF, and HTN.

	Coronary Artery Disease	Congestive Heart Failure	Hypertension
ASA + ACE-I	Depends on trial: No side effects and reduction in mortality (n=19394) [14] vs. increase in mortality (n=6090) [15]	Reduction of clinical benefit (n=2569) [16, 17], increased number of hospitalizations (n=279 and n=1587) [18]	Dose-dependent: low doses of ASA do not affect RR; high doses (>300mg/d) may result in development of resistant hypertension (n=52) [1]. Reduction in cardiovascular events (n=18790) [19]
ASA + ARB	No side effects (n=41267) [20]	No side effects (n=41267 and n=4576) [20, 21]	Reduction in mortality (n=9193) [22], no changes in RR values (n=17) [23]
ASA + MRA	Reduction in mortality (n=6642) [24, 25]	Reduction in mortality (n=6642) [24, 25]	No changes in RR values, no data regarding mortality (n=17) [23]

In a study conducted by Guazzi et al. [1], it is demonstrated that low-doses of aspirin do not significantly affect the RR control in hypertensive patients treated with ACE inhibitor and the dose of 300 mg significantly reduces the antihypertensive effect of ACE inhibitors, but only in some patients. This subpopulation was characterized by a significantly higher incidence of severe hypertension and increased baseline activity of the RAA system. However, other studies do not confirm these observations, while highlighting the significant reduction in cardiovascular events in HTN patients taking ASA, which was reflected in the results of the HOT study [90]. Inhibiting of the renal prostaglandin production and decreased NO-bioavailability in kidneys by the NSAIDs and ASA at high doses may result in development of resistant hypertension [19].

An interesting study to verify the existence of the ACE-I class effect in the effect on the prostanoids' synthesis was conducted by Rodriguez-Garcia et al. The authors compared the effect of captopril, enalapril, ramipril, and fosinopril on the reduction of RR and the daily urinary excretion of the prostanoid pathway final metabolites (6-oxo-PGF-1 alpha and TXB2). Their study also included healthy subjects who did not receive any medications. A significantly lower baseline synthesis of prostacyclin in the study group was shown, and the hypotensive action of ACE-I, secondary to excessive synthesis of PGI, revealed being a class effect [91, 92]. Another small trial showed no reduction of the hypotensive effects of enalapril after administration of 150mg/d ASA [93]. Meune et al. conducted a study which was aiming at determining the minimal dose of ASA capable of limiting the beneficial effects of ACE inhibitors, noting a large interindividual variability in the dose of ASA, which is explained by the different pathogenesis of neurohormonal dysregulation in the vascular wall tension [73].

Proteins whose expression level is altered according to the RR values may be considered as markers of the blood pressure control. Interestingly, the induction of hypertension by an angiotensin II supply in an animal model accelerated aging of platelets and increased oxidative stress. It has been demonstrated that the treatment with angiotensin II receptor blockers increases the antioxidant status of platelets and makes them less susceptible to spontaneous activation in response to agonists including arachidonic acid [94].

There are many studies that describe the interaction of aspirin with ACE inhibitors. Much less data confirms the effects of coadministration of ASA and ARB. One of the studies, based on a LIFE (Losartan Intervention For Endpoint reduction) trial, indicates the positive effects of coadministration of drugs on reducing the frequency of MI and other endpoints in patients with hypertension [95]. In addition, the study conducted by Nawarskas et al. showed no differences in the values of mean as well as systolic and diastolic blood pressure during the treatment with only losartan and in its combination with a low (81mg/day) and high (325mg/day) dose of aspirin. The same researchers received similar results when replacing losartan with enalapril [22]. The data summarizing the papers on the outcomes of combined use of ASA and ACE-I, ARB, and MRA in coronary artery disease, congestive heart failure, and hypertension is summarized in Table 1.

8. Conclusions

Prospective clinical studies with hard endpoints revealed no clinically significant negative interaction between ASA at cardioprotective doses and ACE-I, ARB, or MRA. However, it has been shown that the combination of these groups of drugs provides much greater clinical benefits than using them separately. According to some authors, obtaining a synergistic effect and thus greater reduction in mortality can be achieved by the addition to the ACE-I therapy an antiplatelet drug with another, COX-independent mechanism of action [23, 96–99].

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

Simvastatin Effects on Inflammation and Platelet Activation Markers in Hypercholesterolemia

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Background. Beside the lipid-lowering effect, statins slow the progression of atherosclerosis by exerting anti-inflammatory and platelet inhibiting effects. We investigated whether platelet inhibition by simvastatin correlates with the statin effects on lipid lowering, inflammation, oxidative stress, and endothelial and platelet activation. *Methods*. In hypercholesterolemic patients allocated to diet (n=20) or a 2-month treatment with diet plus 40 mg simvastatin (n=25), we evaluated platelet aggregating responses to ADP, collagen, and arachidonic acid (AA), the effect of aspirin on AA-induced aggregation, pro- and anti-inflammatory and atherogenic mediators (IL-1β, -5, -6, -7, -8, -9, -10, -12, and -13, IFN-γ, IP-10, Eotaxin, and sRAGE), markers of endothelium (sE-selectin, VEGF, and MCP-1) and platelet activation (sP-selectin, sCD-40L, RANTES, and PDGF-bb), and oxidative stress (8-OH-2'-deoxyguanosine). *Results*. After treatment, beside the improvement of lipid profile, we observed the following: a reduction of platelet aggregation to ADP (p=0.0001), collagen (p=0.0001), AA (p=0.003); an increased antiaggregating effect of aspirin in the presence of AA (p=0.0001); a reduction of circulating levels of IL-6 (p=0.0034), IL-13 (p<0.0001), IFN-γ (p<0.0001), VEGF (p<0.0001), sE-selectin (p<0.0001), sCD-40L (p<0.0001), sP-selectin (p=0.003), and 8-OH-2'-deoxyguanosine (p<0.0001); an increase of IL-10 and sRAGEs (p=0.0001 for both). LDL-cholesterol levels (i) positively correlated with IL-6, IFN-γ, E-selectin, sCD-40L, 8-OH-2'-deoxyguanosine, platelet aggregation to ADP, collagen, AA, and aspirin IC-50 and (ii) negatively correlated with IL-10 and sRAGE. In multiple regression analyses, LDL-cholesterol was the strongest predictor for most parameters of platelet reactivity. *Conclusion*. In primary hypercholesterolemia, simvastatin treatment reduced platelet activation and subclinical inflammation and improved endothelial dysfunction. LDL-cholesterol levels were the major correlate of platelet reactivity; however, other effects of statins may contribute to reducing the progression of atherosclerosis.

1. Introduction

Inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (statins) are the most relevant drugs used to lower serum cholesterol levels. In chronic therapy, they are highly effective in the prevention of cardiovascular events [1]. Furthermore, they slow the progression of atherosclerosis by mechanisms related not only to the cholesterol lowering effect, but also to the so-called "pleiotropic" effects, including the influence on subclinical chronic inflammation and haemostasis [2–6].

In particular, statins exert anti-inflammatory properties [7] and inhibit that of the proinflammatory cytokines [8]. The anti-inflammatory effect of statins has been attributed to their ability to reduce, by inhibiting HMG-CoA reductase, not only cholesterol synthesis but also the activation of the mevalonate pathway, with the consequent reduction of isoprenylated and geranylgeranylated proteins, and in particular of Ras prenylation. The statin-induced Ras inhibition reduces the activity of the transcription factor nuclear factor kappa B (NF-kB), which is involved in a wide range of inflammatory pathways and in the formation of reactive oxygen species (ROS) [9]. This statement is confirmed by the observation that high dose simvastatin reduces the binding activity of the proinflammatory transcription factor NF-kB and the concentrations of inflammatory molecules, while the combination

of low dose simvastatin with ezetimibe, resulting in a similar low-density lipoprotein (LDL)-cholesterol reduction, does not affect the inflammatory markers [10].

Effects of statins on endothelial function, immunomodulation, and thrombogenesis could play a role in their ability to prevent cardiovascular events, to ameliorate the prognosis of patients affected by acute myocardial infarction, and to reduce the risk of restenosis after coronary angioplasty [11]. In particular, simvastatin has been shown to exert endothelial beneficial effects by promoting nitric oxide production [12], vasorelaxation [13, 14], and improving leukocyte/endothelium interactions [15]. However, it is still debated whether these effects contribute to an additional cardiovascular risk reduction beyond that expected from LDL-cholesterol lowering [11, 16–18].

Links between chronic inflammation and atherothrombosis are very tight [19] and involve platelets, which interact with endothelial cells and leukocytes by adhesion molecules and trigger inflammation by releasing proinflammatory molecules [20–22].

The mechanisms of inflammation-induced thrombosis, including the relationships between inflammation and platelet function, have been reviewed [23].

Platelets derived from patients with elevated plasma LDL-cholesterol show in vitro hyperaggregability, increased fibrinogen binding and surface expression of P-selectin, and increased production of thromboxane A_2 (TXA_2) and of superoxide anion, whereas plasma derived from the same patients contains increased concentrations of platelet activation markers, such as soluble CD40 ligand (sCD-40L) and beta-thromboglobulin [24].

The statin-induced reduction of platelet activity has been associated with changes of LDL-cholesterol, oxidized-LDL, and P-selectin [25].

The relationships between statin effects on inflammatory molecules and platelet function have been addressed by very few studies. A peculiar aspect of the antiplatelet effect of statins is their ability to reduce the so-called "aspirin resistance", a phenomenon associated with adverse cardiovascular outcomes and increased mortality, more frequent in hyperlipidaemic patients than in general population [26]. From the biochemical point of view, elevated cholesterol reduces the aspirin-mediated platelet acetylation, a mechanism involved in the aspirin-induced antiaggregating effect, exerted via the irreversible inhibition of TXA_2 biosynthesis [27]. Therapy with statins is able to significantly reduce platelet TXA_2 formation in patients taking low dose of aspirin [28].

Furthermore, in vitro experiments carried out by platelet incubation with pravastatin or simvastatin and aspirin demonstrate that statins improve the aspirin-induced platelet inhibition, suggesting that they directly interact with platelet membranes (though these interactions do not include statin's effects on membrane cholesterol or membrane permeability) and modulate signalling pathways in platelets [29].

On the other hand, some "in vivo" studies demonstrating a platelet inhibiting effect of statins after only few days of treatment suggest an effect largely independent of cholesterol lowering [25, 30, 31]. Thus, some mechanisms involved in platelet inhibition occur before any reduction of cholesterol concentrations.

In summary, literature data show that statins exert a platelet antiaggregating effect, but it is still not clear whether it is mediated by statin effects on LDL-cholesterol, or inflammation, or both, or by direct statin effects on platelets.

We designed this study to investigate the association between the platelet inhibitory effect of simvastatin with the lipid lowering and a wide spectrum of pro- and anti-inflammatory, pro- and anti-atherogenic markers induced by the drug in patients affected by primary hypercholesterolemia.

2. Methods

2.1. Subjects, Materials, and Methods. We investigated forty-five patients affected by newly diagnosed primary hypercholesterolemia. They did not have a family history of diabetes mellitus and were otherwise healthy on the basis of medical history, physical examination, and standard diagnostic procedures; in particular, they did not present arterial hypertension, impaired fasting glucose, or impaired glucose tolerance measured by the oral glucose tolerance test (OGTT), congestive heart failure, previous peripheral or coronary or cerebral ischemic vascular diseases, endocrine diseases (including hypothyroidism), renal, hepatic, or hepatobiliary diseases, and myopathic or haemostatic disorders. From the study, we also excluded patients on treatment with nonsteroidal anti-inflammatory or antiplatelet drugs, or antioxidant supplements in the previous three weeks. Patients were randomized to be treated with diet plus simvastatin 40 mg/die for two months (n=25) or diet alone without pharmacological intervention (n=20). All patients followed a low-fat diet close to the Adult Treatment Panel (ATP) III guidelines (7% energy from saturated fat and 200 mg dietary cholesterol per day). The study was approved by the Ethics Committee of San Luigi Gonzaga Hospital and all participants authorized data use for investigational purpose by signed informed consent. At baseline and after two months, all subjects were submitted to a clinical evaluation and in fasting venous blood samples we assessed the following parameters.

(A) Metabolic Parameters and Insulin. Glucose, total and HDL-cholesterol, triglycerides, and apolipoprotein B (Apo B)-100 were measured by automated chemistry by the Central Laboratory of our hospital. LDL-cholesterol was calculated according to the Friedwald's formula. Insulin was measured by a radioimmunoassay kit (Biochem Immuno System, Bologna, Italy). Insulin sensitivity in the fasting state was estimated using HOMA-IR index by the following formula: fasting plasma glucose (mmol/l) x fasting serum insulin (μU/ml) divided by 22.5. HOMA-IR is commonly used in clinical studies as a reliable marker of insulin resistance [32].

(B) Markers of Inflammation, Endothelial and Platelet Activation, and Oxidative Stress. They are as follows: (i) proinflammatory, proatherogenic cytokines and chemokines: Interleukin-1β (IL-1β), Interleukin-5 (IL-5), Interleukin-6

(IL-6), Interleukin-7 (IL-7), Interleukin-8 (IL-8), Interleukin-9 (IL-9), Interleukin-12 (IL-12), Interleukin-13 (IL-13), Interferon-Inducible Protein (IP-10), Interferon-γ (IFN-γ), Eotaxin, and Monocyte Chemoattractant Protein-1 (MCP-1); (ii) anti-inflammatory and antiatherogenic markers: Interleukin-10 (IL-10) and Soluble Receptor of Advanced Glycation End Products (sRAGE); (iii) markers of endothelial activation: Vascular Endothelial Growth Factor (VEGF) and soluble E-selectin (sE-selectin); (iv) markers of in vivo platelet activation: soluble P-selectin (sP-selectin), sCD-40L, Platelet Derived Growth Factor-BB (PDGF-BB), and RANTES; (v) a marker of in vivo oxidative stress: 8-hydroxy-2'-deoxyguanosine (8-OHdG).

Serum and plasma samples for biomarkers detection were stored at -80°C until assayed.

Serum concentrations of IL-1β, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13 IFN-γ, IP-10, Eotaxin, MCP-1, VEGF, RANTES, and PDGF-bb were measured in duplicate by using the Bio-Plex cytokine assay (Bio-Rad Laboratories Inc., Hercules, CA, USA) according to manufacturer's instructions. The Bio-Plex system combines the principle of a sandwich immunoassay with the Luminex fluorescent-bead-based technology allowing the simultaneous measurement of many cytokines.

Serum levels of sE-selectin, sP-selectin, sRAGE, and sCD40L were measured in duplicate with enzyme-linked immunosorbent assay kits (R&D Systems, Abingdon, United Kingdom) according to manufacturer's instructions.

Serum levels of 8-OH-dG were measured by a competitive enzyme-linked immunosorbent assay (Bioxytech 8-OHdG-EIA, OXIS Health Products, Portland, Oregon).

(C) Platelet Function Assays. (i) Platelet aggregation: venous blood samples were withdrawn without stasis and anticoagulated with 3.8% sodium citrate, pH 7.4 (1ml for 9 ml of blood). Platelet-rich plasma (PRP) was obtained by using the Platelet Function Centrifuge (BioData Corporation, Horsham, PA), designed to provide a rapid separation of PRP by a centrifugation for 30 sec. From the top, only two-thirds of the supernatant were collected to avoid contamination by other circulating cells and the remaining blood was further centrifugated for 180 sec to obtain platelet-poor plasma (PPP).

PRP samples were stimulated by arachidonic acid (AA) (1mmol/l), ADP (10 μmol/l), and collagen (4 mg/l) (Mascia Brunelli, Milan, Italy) and platelet aggregation was measured as light-scattering changes by using an eight-channel aggregation system (Platelet Aggregation Profiler, PAP-8, BioData Corporation) according to the Born's method [33]. Platelet aggregation in response to agonists was reported as maximal aggregation (MA). Each aggregation test was recorded for 5 min after the addition of the agonist.

(ii) Platelet sensitivity to aspirin: PRP obtained as described above was stimulated by AA (1 mmol/l) in the absence (see above) and in the presence of a 30 min preincubation with lysine acetylsalicylate (L-ASA) (1-50 μmol/l) (Sanofi-Aventis, Milan, Italy). Platelet aggregation was measured as described above.

2.2. Statistical Analysis. Data are expressed as mean ± SD. Normality of data was checked using Shapiro–Wilk test. Continuous data was examined using parametric analyses performed by Student's t-test for paired and unpaired data. Data are given as mean ± standard deviation (SD). Data with a non-Gaussian distribution were analysed using the Mann–Whitney U test and Wilcoxon signed-rank test, as appropriate. Univariate linear regression analysis was performed to assess the correlation of lipid parameters with circulating biomarkers and platelet aggregation. Pearson's correlation was used to examine the significance of correlation between variables. To evaluate the combined effects of different variables on the platelet parameters, we used a multivariate linear regression model with a backward approach. All analyses were performed with SPSS v.24.

3. Results

The clinical characteristics of the investigated subjects at baseline and after two months with or without simvastatin therapy are shown in Table 1. At baseline, anthropometric and clinical and metabolic parameters did not significantly differ between the two groups. After two months, in the group of patients treated with simvastatin we found, as expected, a significant reduction of total and LDL-cholesterol and of Apo B-100, whereas no differences were found in control group.

3.1. Changes in Inflammatory, Atherogenic, and Oxidative Stress Markers. Table 2 shows circulating levels of inflammatory, atherogenic, and oxidative stress markers before and after the two months of follow-up. At baseline no differences were found between the two groups for each investigated biomarker. After two months, in simvastatin-assigned group, significant reductions of IL-6 (27%, $p<0.01$), IL-8 (14%, $p<0.05$), IL-13 (38%, $p<0.0001$), IFN-γ (52%, $p<0.0001$), and 8-OH-dG (28%, $p<0.0001$) and significant increases of IL-10 (104%, $p<0.0001$) and sRAGE (55%, $p<0.0001$) were found. In control group, all these parameters did not change.

3.2. Changes in Endothelial and Platelet Activation Markers. A two-month treatment with simvastatin induced a significant decrease of the endothelial dysfunction markers sE-selectin (33%, $p<0.0001$) and VEGF (30%, $p<0.0001$) (Figure 1). As far as platelet activation markers are concerned, we observed a significant reduction of sP-selectin (21%, $p=0.003$) and sCD-40L (41%, $p<0.0001$) whereas RANTES and PDGF-BB did not change (Figure 2). In patients who served as controls, no difference for each investigated parameter was found.

3.3. Changes in Platelet Aggregability. As shown in Figure 3, when platelet aggregation tests were evaluated, a two-month treatment with simvastatin resulted in a decrease of MA values in response to ADP (22%, $p<0.0001$), collagen (21%, $p<0.0001$), and AA (19%, $p<0.0001$). In the same subjects, an improvement of the platelet sensitivity to the antiaggregating effects of aspirin was also observed as mirrored by the decrease of L-ASA IC-50 in the presence of AA (65%,

Table 1: Clinical characteristics of hypercholesterolemic patients at baseline and after two months of treatment with diet alone (control) or simvastatin.

	Control (n=20)			Simvastatin (n=25)		
	Before	**After**	**p-value**	**Before**	**After**	**p-value**
Male/Female	M=9 / F=11	-	-	M=11 / F=14	-	-
Age (years)	55±11	-	-	59±13	-	-
BMI (kg/m^2)	25±3	25±3	0.4913	25±4	25±4	0.3698
TC (mg/dl)	270±43	269±50	0.8300	282±30	185±27	<0.0001
HDL-C (mg/dl)	47±5	48±4	0.1523	48±9	48±7	0.3851
LDL-C-C (mg/dl)	188±45	185±51	0.7584	196±33	107±25	<0.0001
APO B-100 (mg/dl)	157±17	155±16	0.3153	152±17	93±15	<0.0001
TG (mg/dl)	177±44	175±48	0.7146	190±61	149±43	0.0027
FG (mg/dl)	87±9	85±8	0.3372	88±9	88±8	0.8580
HOMA-IR	2.6±1.3	2.5±1	0.4331	2.6±1.2	2.4±1	0.9678
SBP (mm Hg)	123±7	124±7	0.7199	126±12	125±10	0.2075
DBP(mmHg)	81±6	80±4	0.7239	79±7	79±7	0.9715

Data are presented as mean±SD. TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; APO B, apolipoprotein B; TG, triglycerides; FG; fasting glucose; SBP, systolic blood pressure; DBP, diastolic blood pressure. For p value, paired Student's t-test or Wilcoxon test was used as appropriate.

Table 2: Pro- and anti-inflammatory and oxidative stress markers in subjects at baseline and after two months of treatment with diet alone (control) or simvastatin.

	Control (n=20)			Simvastatin (n=25)		
	Before	**After**	**p value**	**Before**	**After**	**p value**
Pro-inflammatory						
IL-1b	0.98±0.63	0.94±0.46	0.5008	0.86±0.45	0.79±0.41	0.3625
IL-5	1.8±0.7	1.6±0.7	0.1043	2.0±1.7	2.2±1.4	0.1742
IL-6	6.7±2.5	6.7±2.4	0.6404	6.6±3.4	4.8±2.2	0.0034
IL-7	5.9±2.1	5.7±1.0	0.6676	5.6±2.5	4.9±1.9	0.2143
IL-8	16.8±4.3	15.6±4.0	0.1919	18.9±6.0	16.3±6.7	0.0447
IL-9	18.5±6.6	17.8±7.6	0.4428	17.7±11.6	19.0±15.2	0.6186
IL-12	22.8±7.3	21.4±6.8	0.5124	22.0±13	22.8±13	0.7145
IL-13	5.9±2.2	6.0±2.8	0.6864	7.3±4.3	4.5±2.7	<0.0001
IFN-γ	32.7±14.4	34.0±16.2	0.6744	38.1±13.6	18.3±7.5	<0.0001
IP-10	519±226	476±195	0.1960	491±219	521±264	0.6189
Eotaxin	177±63	177±46	0.7509	143±67	167±54	0.0534
MCP-1	57±23	60±16	0.5257	60±45	50±31	0.1906
Anti-Inflammatory						
IL-10	1.07±0.55	1.04±0.51	0.7046	1.03±0.56	2.10±0.51	<0.0001
sRAGE	678±268	659±248	0.4637	740±263	1147±310	<0.0001
Oxidative Stress						
8-OH-dG	1.60±0.56	1.62±0.58	0.9038	1.84±0.59	1.33±0.56	<0.0001

Data are presented as mean ± SD. IL, interleukin; MCP, Monocyte Chemoattractant Protein; sRAGE, Soluble Receptor of Advanced Glycation End Products; 8-OH-dG, 8-hydroxy-2'-deoxyguanosine. Concentrations are expressed as pg/ml, except where otherwise indicated. For p value, paired Student's t-test or Wilcoxon test was used as appropriate.

p=0.0001). In patients who served as controls, no difference for each investigated platelet function parameter was found.

3.4. Correlation Studies. As shown in Table 3, univariate regression analysis between lipid profile and all circulating markers and aggregability parameters of all patients at baseline revealed that LDL-cholesterol levels are (i) positively associated with IFN-γ, IL-6, VEGF, E-selectin, and sCD-40L as circulating markers of inflammation, endothelial dysfunction, and *in vivo* platelet activation, respectively, 8-OH-dG as marker of oxidative stress, platelet aggregation to ADP, collagen, AA, and IC-50 L-ASA and (ii) inversely associated with the anti-inflammatory markers IL-10 and sRAGE. No significant correlations were found for triglycerides levels (data not shown). Interestingly, when a similar analysis was carried out on the data after simvastatin we found a positive

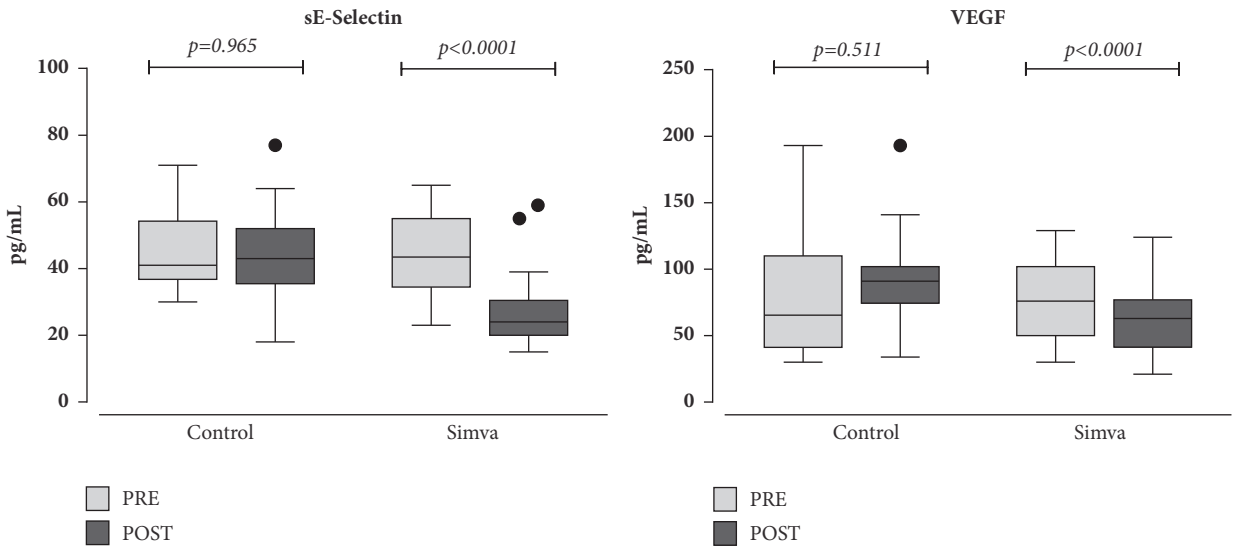

Figure 1: Box-plot analysis of the endothelial dysfunction markers E-selectin and vascular endothelial growth factor (VEGF) in hypercholesterolemic patients assigned to diet alone (Control) or to diet plus simvastatin (Simva) at baseline (pre) and after a two-month follow-up (post). Significance of intragroup differences was estimated by paired t-test or Wilcoxon test, as appropriate. Solid lines: median values; boxes: interquartile range; whiskers: nonoutlier range; closed circles: outliers.

Table 3: Correlation between LDL-C and pro- and anti-inflammatory, oxidative stress, and platelet activation markers in hypercholesterolemic patients at baseline.

	LDL-C	
	r	**p**
IFN-γ	0.493	0.001
IL-6	0.427	0.003
VEGF	0.301	0.044
E-Selectin	0.560	<0.0001
sCD-40L	0.348	0.019
IL-10	-0.364	0.014
sRAGE	-0.484	0.001
8-OH-dG	0.307	0.040
MA ADP	0.638	<0.0001
MA COLL	0.614	<0.0001
MA AA	0.623	<0.0001
IC-50 L-ASA	0.600	<0.0001

IFN, interferon; IL, interleukin; VEGF, vascular endothelial growth factor; sCD-40L, soluble CD-40 ligand; IL, interleukin; sRAGE, Soluble Receptor of Advanced Glycation End Products; 8-OH-dG, 8-hydroxy-2'-deoxyguanosine; MA, maximal aggregation; Coll, collagen; AA, arachidonic acid; IC-50, half-maximal inhibitory concentration; L-ASA, lysine acetylsalicylate.

correlation between LDL-cholesterol and E-selectin (r=0.616, p=0.001) and 8-OH-dG (r=0.500, p=0.011).

Correlation between platelet aggregability and the investigated pattern of circulating biomarkers in all patients at baseline is shown in Table 4. Of note, independently of proaggregating stimulus, an increased platelet aggregation and a reduced antiaggregating effect of aspirin were associated with increase of E-selectin, IFN-γ, and IL-6, thus pointing out the relationships between impaired endothelial function, inflammation, and platelet hyperaggregability. Interestingly, in this context also reduced levels of the anti-inflammatory sRAGE played a role in increasing platelet response to activators (ADP, AA) and decreasing the inhibitory effect of L-ASA.

When the differences between values at baseline and after two months of simvastatin treatment (delta values) of LDL-cholesterol were correlated with the delta values of all the evaluated parameters, no significant correlation was found (data not shown).

Table 5 shows the multiple linear regression analysis with MA to ADP, collagen, and AA and with L-ASA IC-50 to AA entered as dependent variables and the parameters significantly correlated with them (see Table 4) entered as independent variables.

The significant predictors were for MA to ADP, LDL-cholesterol, VEGF, p-selectin, sRAGE; for MA to collagen, LDL-cholesterol alone; for MA to AA, LDL-cholesterol, IL-1b, IL-10, and sCD40L; and for L-ASA IC-50 to AA, LDL-cholesterol and sCD40L.

4. Discussion

This study, carried out in patients with primary hypercholesterolemia, shows that a two-month therapy with simvastatin improved not only, as expected, the lipid profile but also a wide pattern of proatherogenic and prothrombotic parameters. Actually, simvastatin treatment reduced mediators of inflammation, oxidative stress, and endothelial and platelet activation, increased anti-inflammatory circulating markers, reduced platelet aggregating responses to ADP, collagen, and AA and increased platelet sensitivity to aspirin.

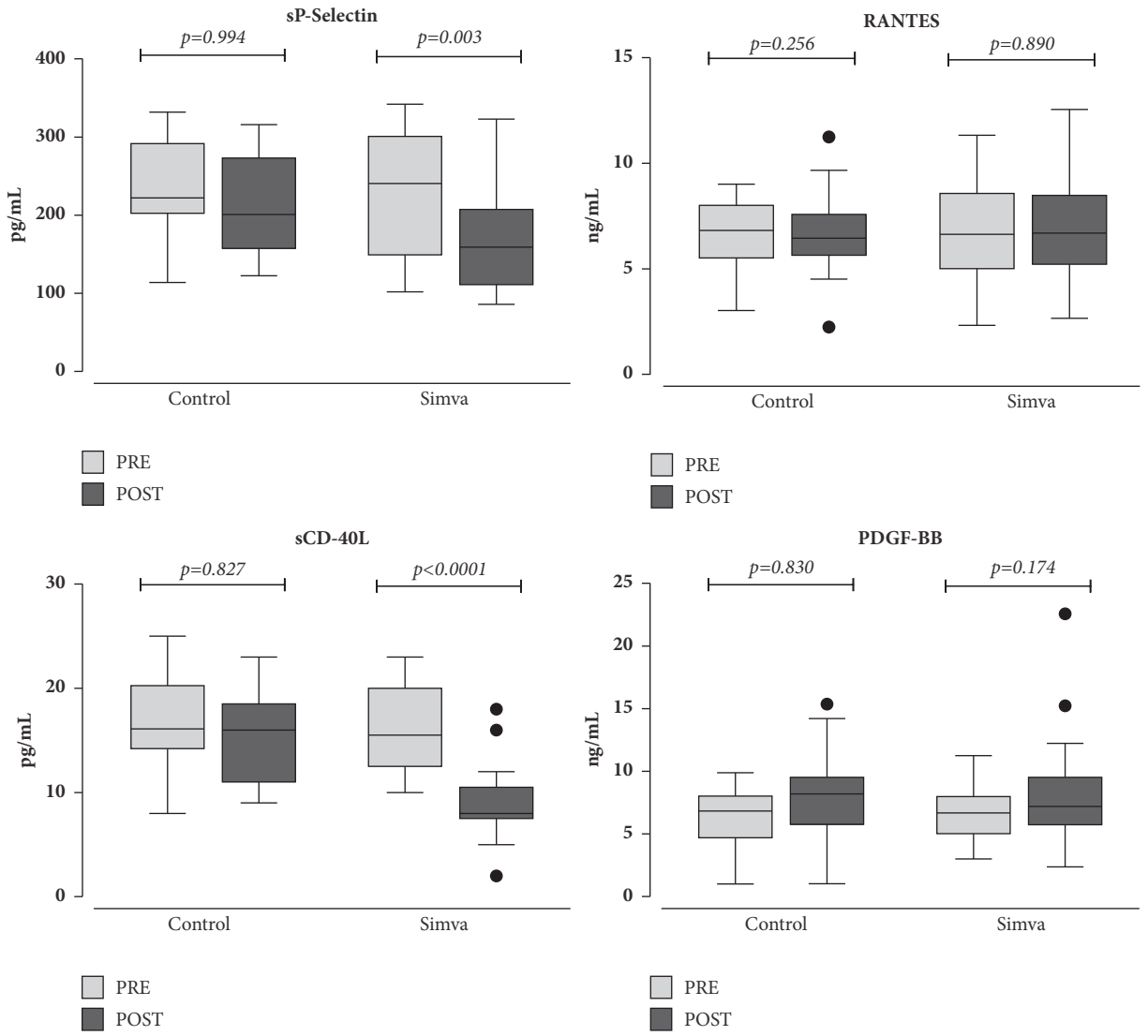

Figure 2: Box-plot analysis of the platelet activation markers soluble P-selectin (sP-selectin), Rantes, soluble CD-40 ligand (sCD-40L), and platelet-derived growth factor (PDGF)-BB, in hypercholesterolemic patients assigned to diet alone (Control) or to diet plus simvastatin (Simva) at baseline (pre) and after a two-month follow-up (post). Significance of intragroup differences was estimated by paired t-test or Wilcoxon test, as appropriate. Solid lines: median values; boxes: interquartile range; whiskers: nonoutlier range; closed circles: outliers.

To the best of our knowledge, this is the first study which simultaneously considered a so wide spectrum of the potential simvastatin effects, trying to correlate them with the platelet effects of the drug.

On the other hand, at baseline, a correlation was observed between LDL-cholesterol levels and markers of inflammation (such as IL-6 and IFN-g), of anti-inflammation (such as IL-10 and sRAGE), endothelial activation markers (such as sE-selectin, VEGF), platelet activation markers (such as sCD-40L), oxidative stress (such as 8-OH-dG), and platelet function parameters evaluated in terms of aggregation to activators (i.e., collagen, AA, and ADP) or response to inhibitor (i.e., L-ASA). The biological explanation of this phenomenon is attributable to the role exerted by LDL-cholesterol on subclinical inflammation, oxidative stress, and platelet activation, as previously mentioned.

The role of LDL-cholesterol on platelets is suggested by evidence showing that lipoprotein disorders affect platelet function. Actually, LDL particles sensitize platelets by the binding of apoB-100 to the specific receptor on the platelet membrane and the subsequent modification of platelet function via a wide spectrum of interactions: in particular, LDL particles in their native form induce hypersensitivity of platelets to agonists resulting in increased aggregation and secretion responses whereas, after oxidation, they become independent platelet activators in stirred platelet suspensions [34].

In our study, when platelet function parameters were correlated with biomarkers at baseline, we observed the occurrence of significant correlations not only with LDL-cholesterol, but also with proinflammatory (such as IL-1b, IL-6, and IFN-γ), anti-inflammatory (such as IL-10 and

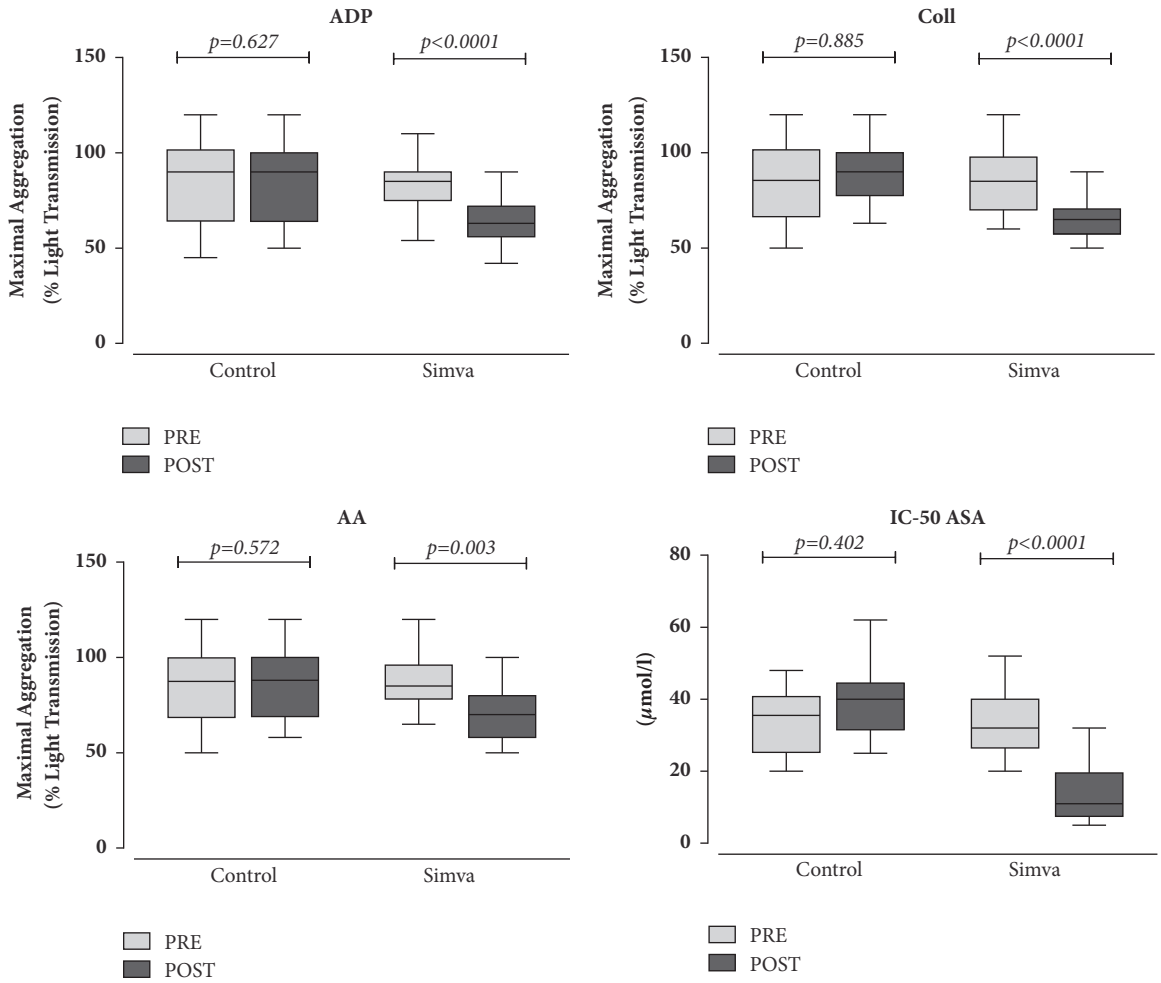

Figure 3: Box-plot analysis showing maximal aggregation to ADP, collagen, arachidonic acid (AA), and lysine acetylsalicylate (L-ASA) half-maximal inhibitory concentration (IC-50) in the presence of AA in hypercholesterolemic patients assigned to diet alone (Control) or to diet plus simvastatin (Simva) at baseline (pre) and after a two-month follow-up (post). Significance of intragroup differences was estimated by paired t-test or Wilcoxon test, as appropriate. Solid lines: median values; boxes: interquartile range; whiskers: nonoutlier range.

sRAGE), endothelial activation (such as sE-selectin, VEGF), and platelet activation (sP-selectin, sCD-40L, and PDGF-BB) markers. In the multivariate analysis, LDL-cholesterol was the parameter that more strongly influenced platelet sensitivity to activators (ADP, collagen, and AA) and inhibitor (aspirin) suggesting a primary role for LDL-cholesterol in determining the cascade of inflammatory events responsible also for the activated platelet function.

In our study, lack of correlations between most of biomarkers levels and LDL-cholesterol after simvastatin suggests that some pleiotropic effects of simvastatin may be independent of their effects on LDL-cholesterol levels. However, a positive and significant correlation after simvastatin was found with E-selectin and 8-OH-dG and this fact induces hypothesizing that the improvement of cholesterol levels may alone influence some peculiar aspects of endothelial function and oxidative stress.

On the other hand, we did not observe significant correlations between delta values of LDL- cholesterol and delta values of all the parameters modified by the simvastatin treatment (data not shown). Although this fact is not surprising and fits with other observations in literature, we are aware, however, that the limited number of subjects enrolled in our study could be a possible explanation for the absence of significant correlations between delta values of LDL- cholesterol and delta values of all the parameters modified after statin treatment. However, from the biological point of view, the plausibility of this phenomenon could be also due to the fact that the mechanisms by which statins exert the lipid-lowering and the pleiotropic effects are different, although the first step is the inhibition of HMG-CoA reductase. Furthermore, the demonstration of a direct effect of the statins on platelets observed in "in vitro" experiments [29, 35] further increases the complexity of the picture.

Of course, the interplay between inflammation and thrombosis is enhanced in the presence of dyslipidemia: not surprisingly, statins, which reduce both lipid concentrations and inflammation, are considered antithrombotic drugs, as reviewed [36, 37].

Table 4: Correlation between platelet function, expressed as maximal aggregation (MA) to ADP (a), collagen (b), AA, and L-ASA IC-50 (d), and pro- and anti-inflammatory, oxidative stress, and platelet activation markers in hypercholesterolemic patients at baseline.

(a) MA to ADP

	r	p
IFN-γ	0.391	0.008
IL-6	0.384	0.009
VEGF	0.326	0.029
P-Selectin	0.326	0.029
E-Selectin	0.432	0.003
sRAGE	-0.453	0.002

(b) MA to collagen

	r	p
IFN-γ	0.326	0.029
IL-6	0.327	0.028
PDGF-BB	0.331	0.027
E-Selectin	0.419	0.004

(c) MA to AA

	r	p
IFN-γ	0.315	0.035
IL-10	-0.448	0.002
IL-1b	0.320	0.032
IL-6	0.425	0.004
E-Selectin	0.515	0.0003
sCD-40L	0.562	<0.0001
sRAGE	-0.515	0.0003

(d) L-ASA IC-50 to AA

	r	p
IL-6	0.347	0.020
E-Selectin	0.339	0.023
sCD-40L	0.442	0.002
sRAGE	-0.454	0.002

TC, total cholesterol; LDL-C, low-density lipoprotein; IFN, interferon; IL, interleukin; VEGF, vascular endothelial growth factor; sCD-40L, soluble CD-40 ligand; IL, interleukin; sRAGE, Soluble Receptor of Advanced Glycation End Products; PDGF, Platelet Derived Growth Factor; Coll, collagen; AA, arachidonic acid; IC-50, half-maximal inhibitory concentration; L-ASA, lysine acetylsalicylate.

The so-called "pleiotropic effects" of statins on endothelial function, vascular inflammation, immunomodulation, and thrombogenesis could play a role in their ability to prevent cardiovascular events, to ameliorate the prognosis of patients affected by acute myocardial infarction, and to reduce the risk of restenosis after angioplasty, as reviewed [17], even if it is still debated whether these effects contribute an additional cardiovascular risk reduction beyond that expected from LDL-cholesterol lowering [11, 16–18].

A previous study demonstrated that simvastatin inhibits TXA_2 biosynthesis and platelet function in hypercholesterolemic patients [38, 39]. Also other statins reduce platelet function. In particular, fluvastatin therapy reduced platelet cholesterol/phospholipid molar ratio and platelet aggregation, suggesting that its antiaggregating effect is due to reduced platelet cholesterol content [40]. Atorvastatin therapy reduced collagen-induced platelet aggregation [41], platelet activation evaluated by flow cytometry [42], and platelet function in patients with coronary heart disease [43]. Pravastatin, as well as simvastatin, reduced platelet thrombus formation after only 8 weeks of therapy [44]. The statin-induced reduction of platelet activity has been associated with changes of LDL-cholesterol, oxidized-LDL, and P-selectin [25]. Our study extends previous studies by showing the inhibiting effects of simvastatin treatment on pathways of platelet aggregation activated by three different agonists and on the L-ASA antiaggregating effect. Furthermore, we observed that simvastatin reduced sCD40L and sP-selectin levels as markers of platelet activation.

As previously mentioned, statins are able to reduce the so-called "aspirin resistance", a phenomenon associated with adverse cardiovascular outcomes and increased mortality [26]. Actually, in our study simvastatin therapy showed the ability to significantly improve the in vitro platelet sensitivity to the antiaggregating effects of aspirin, a finding consistent with data obtained by other authors showing a reduced TXA_2 formation in patients treated with simvastatin and receiving aspirin before and after statin administration [28].

In the present study we observed an effect of simvastatin treatment on a variety of markers of inflammation and we evaluated the relationships between the statin effects on inflammatory molecules and platelet function, an aspect addressed by few studies. It has been shown that eight weeks of treatment with atorvastatin and rosuvastatin are associated with comparable reductions in LDL-cholesterol, high sensitive C reactive protein (hsCRP), 11-dehydro-TXB_2 (a marker of TXA_2 biosynthesis), and 8-iso-prostaglandin $F_{2\alpha}$ (a marker of lipid peroxidation with platelet-activating properties): in this study, in multiple regression analyses, only hsCRP and LDL-cholesterol were independent predictors of 11-dehydro-TXB_2, while only LDL-cholesterol predicted 8-iso-$PGF_{2\alpha}$ [45]. Notably, we showed for the first time that simvastatin treatment is also able to significantly reduce circulating sRAGE concentrations. It is known that sRAGE levels are reduced in hypercholesterolemic patients as compared to healthy subjects and inversely correlate with urinary excretion of isoprostanes and plasma asymmetric dimethylarginine suggesting that the ligand-RAGE axis may link endothelial dysfunction with oxidative stress [46].

5. Conclusions

In conclusion, this study provides the first evidence that a short-term treatment with simvastatin simultaneously affects a wide range of markers of inflammation and atherothrombosis, adding a piece of information to better clarify the rationale of simvastatin therapy in patients at a high cardiovascular risk. In fact, simvastatin therapy, beside its hypocholesterolemic effect, (i) decreases oxidative stress, proatherogenic and proinflammatory markers, (ii) increases antiatherogenic and anti-inflammatory markers, (iii) reduces platelet aggregation to physiological agonists, and (iv) increases platelet

Table 5: Multivariate regression analysis between baseline platelet function values, expressed as maximal aggregation (MA) to ADP (a), collagen (b), AA, and L-ASA IC-50 (d), entered as dependent variable, and markers of lipid control, inflammation, endothelial dysfunction, oxidative stress, and platelet activation markers, entered as independent variables. The table shows the final regression parameters after a backward selection algorithm in which all variables were present in the first step.

(a) MA to ADP

MA to ADP	Adjusted R^2=0.362	F=25.954	p<0.0001
	Constant	B=7.072	p=0.7299
	LDL-C	B=0.250	p=0.0016
	VEGF	B=0.191	p=0.0081
	p-Selectin	B=0.134	p=0.0009
	sRAGE	B=-0.021	p=0.0510

(b) MA to collagen

MA to Collagen	Adjusted R^2=0.393	F=15.239	p<0.0001
	Constant	B=31.722	p=0.0069
	LDL-C	B=0.290	p<0.0001

(c) MA to AA

MA to AA	Adjusted R^2=0.615	F=18.580	p<0.0001
	Constant	B=21.873	p=0.0946
	LDL-C	B=0.211	p=0.0003
	IL-10	B=-8.762	p=0.0240
	IL-1b	B=9.012	p=0.0128
	sCD-40L	B=1.549	p=0.0013

(d) L-ASA IC-50 to AA

L-ASA IC-50 to AA	Adjusted R^2=0.394	F=15.302	p<0.0001
	Constant	B=1.406	p=0.8282
	LDL-C	B=0.132	p=0.0002
	sCD-40L	B=0.609	p=0.0398

sensitivity to the antiaggregating effects of aspirin. In this scenario, LDL-cholesterol levels are a major correlate and possibly a determinant of enhanced platelet reactivity suggesting a primary role for LDL-cholesterol in determining the cascade of inflammatory events responsible also for the impaired platelet function.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors have no conflicts of interest.

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Research Article

Computing of Low Shear Stress-Driven Endothelial Gene Network Involved in Early Stages of Atherosclerotic Process

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Background. In the pathogenesis of atherosclerosis, a central role is represented by endothelial inflammation with influx of chemokine-mediated leukocytes in the vascular wall. Aim of this study was to analyze the effect of different shear stresses on endothelial gene expression and compute gene network involved in atherosclerotic disease, in particular to homeostasis, inflammatory cell migration, and apoptotic processes. *Methods*. HUVECs were subjected to shear stress of 1, 5, and 10 dyne/cm^2 in a Flow Bioreactor for 24 hours to compare gene expression modulation. Total RNA was analyzed by Affymetrix technology and the expression of two specific genes (CXCR4 and ICAM-1) was validated by RT-PCR. To highlight possible regulations between genes and as further validation, a bioinformatics analysis was performed. *Results*. At low shear stress (1 dyne/cm^2) we observed the following: (a) strong upregulation of CXCR4; (b) mild upregulation of Caspase-8; (c) mild downregulation of ICAM-1; (d) marked downexpression of TNFAIP3. Bioinformatics analysis showed the presence of network composed by 59 new interactors (14 transcription factors and 45 microRNAs) appearing strongly related to shear stress. *Conclusions*. The significant modulation of these genes at low shear stress and their close relationships through transcription factors and microRNAs suggest that all may promote an initial inflamed endothelial cell phenotype, favoring the atherosclerotic disease.

1. Background

The development of atherosclerosis is commonly associated with high levels of lipids in blood able to generate the development of sclerotic plaques. New knowledge from basic and experimental science has demonstrated the role for inflammatory pathway in early events of disease pathogenesis. Different in vivo animal models of atherosclerosis show signs of inflammation, both at local than at systemic levels, associated with lipid accumulation in the artery wall. For example, leukocytes, important mediators of host defenses and inflammation, localize in earliest lesions in animal models and in humans as well [1]. The molecular mechanisms involved in lymphocytes recruitment by activated endothelial cells at atherosclerotic lesion formation sites are similar to those reported for neutrophils and monocytes [1, 2]. The different steps of atherogenic leukocyte recruitment such as rolling, adhesion, and transmigration are controlled by functionally specialized chemokines [3, 4]. Whereas soluble chemokines were first described to induce directed chemotaxis of leukocytes, chemokines trigger the integrin-mediated arrest of rolling leukocytes [5, 6]. Chemokine/chemokine receptor network is essential for direction of leukocyte migration in homeostatic and inflammatory conditions. Numerous reports described the important role of chemokines and of their receptors in the regulation of leukocyte recruitment during atherosclerosis [7]. Chemokine (C-X-C motif) Receptor 4 (CXCR4) signaling has been reported to modulate cell chemotaxis, survival [1, 8], and apoptosis [9–11]. Through this receptor the Macrophage Migration Inhibitory Factor

(MIF), able to regulate inflammatory cell (T cells, monocytes) recruitment to lesion area [12, 13], expresses its proatherogenic action.

Involved in monocyte adhesion is also Intercellular Adhesion Molecule 1 (ICAM-1). Upon cytokine stimulation with Tumor Necrosis Factor alpha (TNF-alpha) [14], Angiotensin II, and Oxidized Low Density Lipoprotein (ox-LDL) [15], ICAM-1 expression increases in wall vessel cells [16, 17] producing proinflammatory effects with leukocyte recruitment.

Although the pathobiology of atherosclerosis is a complex biological multifactorial process, blood flow-induced shear stress has emerged as essential feature of atherogenesis. This fluid drag force, acting on vessel wall, is mechanotransduced into biochemical signals, resulting in vascular behavior changes. Maintenance of physiologic, laminar shear stress is known to be crucial for normal vascular functioning, which includes regulation of vascular caliber, inhibition of proliferation, thrombosis, and inflammation of the vessel wall. Nonlaminar flow promotes changes in endothelial gene expression, cytoskeletal arrangement, leukocyte adhesion, and vasoreactive, oxidative, and inflammatory states of artery [5, 18, 19]. Disturbed shear stress also influences site selectivity of atherosclerotic plaque formation and its associated vessel wall remodeling, which can affect plaque vulnerability and stent restenosis. Shear stress is critically important in regulating atheroprotective normal physiology as well as pathobiology and dysfunction of vessel wall through complex molecular mechanisms that promote atherogenesis [20].

The aim of this work is to analyze the effect of different shear stress levels in vitro on endothelial gene expression using a laminar flow bioreactor [21]. Thanks to microarray analysis, the possible involvement in early phases of atherosclerotic disease of genes significantly regulated and linked to inflammatory and apoptotic process have been observed. In particular, CXCR4, ICAM-1, Tumor Necrosis Factor Alpha-Induced Protein 3 (TNFAIP3), and Caspase-8 (CASP8) genes that present a role in atherosclerosis development at different level and with different functions were considered. With the support of bioinformatics analysis it is possible to highlight existing relationships between genes under investigation generating a potential regulation system network.

2. Methods

2.1. Endothelial Cell Culture. Primary Human Umbilical Vein Endothelial Cells (HUVECs) were isolated from human cords. Fresh human umbilical cords were recovered from healthy females at the Obstetrics and Gynecology Unit of the Azienda Ospedaliera Universitaria Pisana, after obtaining written informed consent for use of these samples in research approved by the Local Ethics Committee of Area Vasta Nord Ovest. The umbilical cords were stored in PBS at 4°C, sent to our laboratory within 1 hour of delivery, and treated anonymously conforming with the principles outlined in the Declaration of Helsinki. Umbilical vein was cannulated, washed with Phosphate Buffered Saline (PBS) solution, and filled with 3 mg/ml collagenase IV solution in PBS. After 20 min in incubator, it was washed again with Endothelial Cell Growth Medium (ECGM) (Promocell, Heidelberg, Germany) to block collagenase action and, after centrifugation (900 rpm, 5 min), pellet was recovered with fresh complete media and seeded in gelatin 1% pretreated flask for cell adhesion. Every 2 days media culture was changed, until the confluence.

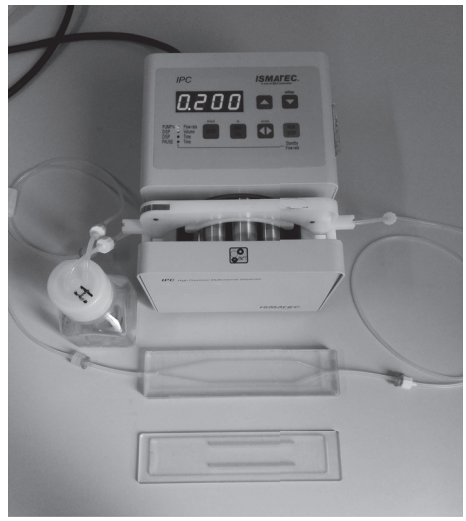

Figure 1: Assembled laminar flow bioreactor (LFB). Bioreactor is composed by a mixing chamber filled with a complete culture media with 5% of Dextran, a cell culture chamber, and a peristaltic pump. All the components were connected in a closed loop and put in an incubator to preserve temperature (37°C) and CO2 concentration in air (5%).

2.2. Bioreactor. The bioreactor system [21, 22] (Figure 1) is composed by a mixing chamber, filled with 12 ml of complete culture media supplemented with 5% of Dextran (Sigma-Aldrich, St. Louis, MO, USA), a cell culture chamber, and a peristaltic pump (ISMATEC, Wertheim, Germany). All the components were connected in a closed loop and assembled system was put in incubator.

In order to obtain a central region with a well-defined and uniform wall shear stress, active cell culture chamber (70x20x2 mm^3) shape was designed by accurate modeling analysis performed with finite element software for simulation of fluid dynamic flow [21].

The chamber is fabricated in polydimethylsiloxane (PDMS, Sylgard 184®) (Dow Corning, Midland, MI, USA), a biocompatible silicone polymer, through mill-molding.

2.3. Bioreactor Experimentation. For experiments, HUVECs between 2^{nd} and 5^{th} passage were used. When at confluence, cells were treated with 0.5% Trypsin (Lonza, Basel, Switzerland). Once detached from flask, endothelial cells were centrifuged at 900 rpm for 5 min. Pellet was resuspended in fresh media; cells were counted and seeded (15000 cells/cm^2) on fibronectin 3 $\mu g/cm^2$ pretreated Thermanox slides (dimensions 2 x 6 cm^2) (NUNC, Rochester, NY, USA).

When HUVECs covered surface slide, experiments with bioreactor started. The system was set in order to furnish different flow conditions, corresponding to shear stress values of 1, 5, and 10 dyne/cm^2. Every experiment had duration of 24 hours; at the end, slides were recovered and cell images acquired under microscope. Then, endothelial cells were trypsinized with 200 μl/slide. Once cells were detached, 1 ml of medium was added to block trypsin action and 50 μl of suspension was recovered, before centrifugation, to perform CellTiter-Blue® Cell Viability Assay (Promega, Madison, USA). This assay is useful for monitoring cell viability through the ability of living cells to convert a dye (resazurin) into a fluorescent end product (resorufin, 579_{ex} /584_{em}).

Finally, residual cell medium was centrifuged and the pellet was resuspended in 50 μl of RNA later solution (Qiagen, Hilden, Germany) and frozen at -20°C to prevent RNA degradation until the genetic analysis.

2.4. Total RNA Extraction. Total RNA has been extracted from HUVECs using RNeasy® Micro Kit QIAGEN for small amounts of human cells ($\leq$ 5 x 10^5cells). Briefly, cell pellets were first lysed and homogenized in a highly denaturing guanidine-isothiocyanate-containing buffer and ethanol, which immediately inactivates RNases to ensure isolation of intact RNA. The lysate was passed through a RNeasyMinElute spin column, where total RNA binds to the membrane and contaminants were washed away. Traces of DNA that may copurify are removed by a DNase treatment.

The RNA quality control was performed with the Agilent BioAnalyzer system (Agilent Technologies, Santa Clara, CA, USA) that separated and subsequently detected RNA samples via laser induced fluorescence detection.

2.5. Microarray Analysis by Affymetrix Technology. 100 ng of total RNA from each experimental set has been amplified resulting in unlabeled cDNA. An in vitro transcription reaction was performed in presence of biotin-labeled ribonucleotides mixture to produce biotinylated cRNA from the cDNA template. Biotinylated cRNA molecules were hybridized to their complementary sequences on the GeneChip surface. For each experimental condition, 2 microarrays (HG-U133-Plus 2.0, Affymetrix, Santa Clara, CA, USA) have been used; every array allows measuring the expression level of over 47,000 human transcripts, representing 38,573 gene clusters in the UniGene database plus 841 anonymous full-length transcripts, and a number of anonymous partial sequences of cDNA. The fluorescence data were processed using MicroArray Suite software version 5.0 (Affymetrix). Data from the gene microarray experiments were preprocessed using the Robust Multiarray Average (RMA) algorithms making adjustments for systematic errors introduced by differences in procedures and dye intensity by collaboration of COGENTECH (Consortium for Genomic Technologies, Milan, Italy). Microarray data have been submitted to the Gene Expression Omnibus (GEO) under accession n. GSE45225.

2.6. Quantitative Real-Time PCR of CXCR4 and ICAM-1. For Real-Time Polymerase Chain Reaction (RT-PCR), the reverse transcription of total RNA (500 ng) was performed using the Transcriptor First strand cDNA synthesis kit (Roche Applied Science, Indianapolis, IN, USA).

Every cDNA solution (2 μl) was mixed with $MgCl_2$ (3 mM), primers (0.3 μM) and Hybridization Probes (0.15 μM), designed ad hoc by TIBMOLBIOL (Roche), and with LightCyclerFastStart DNA Master Mix by Roche (10X). The cycling protocol consisted of denaturation step at 95°C for 10 s, annealing step at 58°C for 10 s and extension step at 72°C for 10 s. This protocol was repeated for 45 times. Fluorescence was detected at 640 nm and measured at the end of each extension step. The LightCycler software 4.05 calculated the Crossing point, (Cp), where the samples fluorescence curve turns sharply upward. This turning point corresponds to the first maximum of the second derivative of the curve. For final quantification of samples, an external calibration curve was obtained using specific amplicon with known concentration.

2.7. Preparation of Standard Curve. For absolute quantification of CXCR4 and ICAM-1 gene expressions, we prepared standard curves amplifying a fragment of CXCR4 or ICAM-1 transcript coming from a sample of cDNA with conventional PCR. The resulting fragments were purified by the QIAquick PCR Purification kit protocol (QIAgen). CXCR4 (46.1 ng/l) and ICAM-1 (25.5 ng/l) products were measured at 260 nm. Sevenfold serial dilutions of quantified ICAM-1 and CXCR4 standard amplicons were used for standard curve preparation (7 points with concentration range from 10^{-4} to 10^{-10} ng/l).

2.8. Statistical Analysis. After quantile normalization and filtering with a false discovery rate below 20% (FDR < 0.2), 6807 genes of 40508 analyzed were selected and clustered by Euclidean distance measure. Genes were sorted for differential expression based on one-way ANOVA and Different Expressed Genes (DEG) were identified as those genes having adjusted p values < 0.05 with Fold Change (FC) $\geq$ 2 fold in modulus.

2.9. Bioinformatics Analysis. In order to further support wet lab results and to obtain a wider understanding of the possible regulations surrounding genes in HUVECs under low shear stress, and their role in the early phases of atherosclerosis, a bioinformatics approach was performed. Regulators include both microRNA and Transcription Factors (TF) whose role is central in posttranscriptional and transcriptional regulation and whose contribution may better uncover the dynamics of the regulatory interaction that take place in the network comprising the already identified genes.

For the network enhancing process, we used ReNE (Regulatory Network Enhancer), a Cytoscape 3.x plugin we developed to automatically enrich a gene-based regulatory network by adding transcriptional, posttranscriptional, and translational data [23]. During the enhancing process, ReNE automatically retrieves regulatory information from multiple public repositories (i.e., miRIAD, TargetMine, miRanda, NCBI, UniProt, TargetHUB, PicTar, miRTarBase, TargetScan,

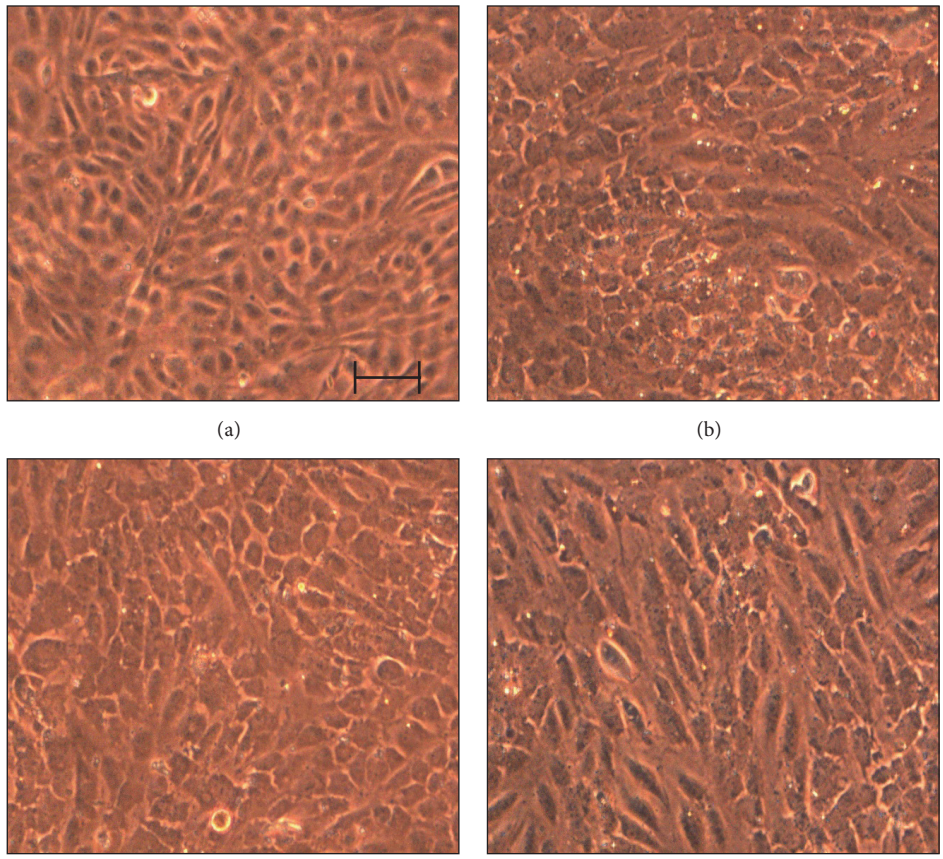

Figure 2: HUVECs under shear stress conditions. Picture of HUVECs subjected at 0 (a), 1 (b), 5 (c), and 10 dyne/cm^2 (d) after 24 hours of stimulus (scale bar equal to 100 μm).

and miRBase) and also provides a set of utilities in order to filter out the large amount of false positive predictions (usually stored in public repositories) for guaranteeing more reliable data.

3. Results

3.1. Flow Dependence of Cell Reorganization and Viability. Endothelial cells were photographed in order to analyze cell shape and behavior at different shear stress levels. One of the first results was the detection of flow-induced modifications on cell morphology, characterized by cytoskeletal reorganization, with prevalent cobblestone shape and initial elongated structure (Figures 2(b), 2(c), 2(d)) with respect to control (Figure 2(a)).

Furthermore, cell viability presented a statistical increase in the case of 1 dyne/cm^2 with respect to 5 and 10 dyne/cm^2, indicating that lowest shear stress conferred proliferative behavior to endothelial cells (Figure 3).

This last result highlights a significant modulation by shear stress of cell activity, in terms of metabolic/proliferating profile but also in genetic modulation of biological mediators of our interest.

3.2. Microarray Results. Microarray data were published on Gene Expression Omnibus (GEO) (access number GSE45225). Globally, 3000 ID probes were found to be differentially expressed in our experimental conditions. HUVECs exposed to different shear stress values were initially compared with those that had been maintained under static conditions to verify how many gene expressions were modulated. As shown in Table 1, the used low shear stress regulated 463 ID probes with respect to static condition, while middle and high shear stress expressed 1008 and 921 different genes, respectively, compared to no flow state.

A comparison between flows may give a more accurate picture of gene expression modulation in physiological or pathological states. Low shear stress, compared to middle and high flow, regulated 142 and 397 different genes, respectively. Among 397 genes of 1 versus 10 dyne/cm^2 stress, 206 (52%) were up- and 191 (48%) were downregulated; in 1 versus 5 dyne/cm^2 comparison 57 (40%) were up- and 85 (60%) were downregulated.

In the comparison between low and high shear stress, we focused on genes that showed the highest up- and downregulation (selection criteria were $p<0.05$ and FC>2).

Microarray analysis (Table 2) highlighted a strong upregulation of CXCR4 at low (1 dyne/cm^2) compared to high shear

Table 1: Number of differentially expressed genes between conditions.

Comparison	Factor considered	Selection criterion	Number of DEG
0 vs 1	Flow effect	p<0.05 & |FC| >2	463
0 vs 5	Flow effect	p<0.05 & |FC| >2	1008
0 vs 10	Flow effect	p<0.05 & |FC| >2	921
1 vs 5	Flow effect	p<0.05 & |FC| >2	142
1 vs 10	Flow effect	p<0.05 & |FC| >2	397

Table 2: Fold variations of genes under investigation with microarray technique.

Probeset ID	Gene	Fold variation 1 vs 5	Fold variation 1 vs 10
209201_x_at	CXCR4	2.76	5.42
217028_at	CXCR4	2.78	5.54
211919_s_at	CXCR4	2.15	4.53
213373_s_at	CASP-8	-	2.13
202643_s_at	TNFAIP3	-2.09	-3.28
202644_s_at	TNFAIP3	-2.71	-4.19
202637_s_at	ICAM-1	-2.10	-3.88
202638_s_at	ICAM-1	-2.05	-3.33

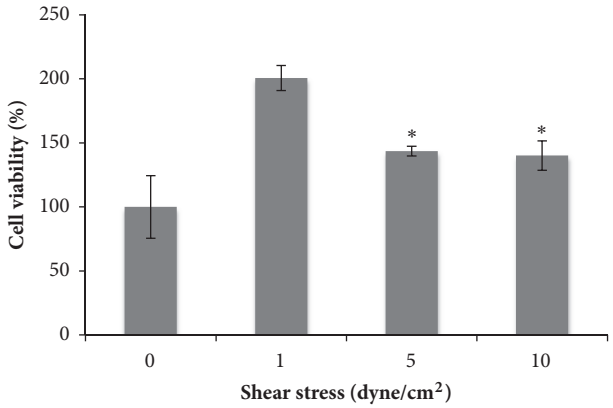

Figure 3: Cell viability of HUVECs after shear stress treatment. (ANOVA statistical analysis p<0,05 for: 1 versus 5; 1 versus 10).

stress (4.53-5.54 fold variation) and significant at intermediate level (2.15-2.78 fold variation) in inversely proportional shear-dependent manner. Caspase-8 gene was significantly upregulated only at low shear stress with respect to 10 dyne/cm^2 (2.13 fold variation). Conversely, a marked downregulation of TNFAIP3 gene (-4.19- and -3.28-fold variation) was observed. A mild reduction in gene expression (-3.33- and -3.88-fold variation) was observed also for ICAM-1 when 1 dyne/cm^2 was compared to 10 dyne/cm^2. In general, low shear stress seems to represent a key factor in gene expression modulation of endothelial response to fluid dynamical forces.

3.3. Quantitative Real-Time PCR Results. Microarray results gave a global vision about the effects of fluid mechanical stimulation on endothelial cell culture. Focusing on the role of chemokines in early stages of atherosclerosis development, gene expressions of CXCR4 and ICAM-1 mediators were also analyzed. Results from RT-PCR confirmed the trend observed for these genes in microarray analysis. ICAM-1 mRNA levels increased from 1 to 10 dyne/cm^2 (Figure 4(a)) while CXCR4 gene expression, high at 1 dyne/cm^2, decreased when compared to 5 and 10 dyne/cm^2 experiments (Figure 4(b)). Both ICAM-1 and CXCR4 mRNA concentrations are very low in static condition.

3.4. Bioinformatics Analysis. We used ReNE to enhance and better understand regulatory relations that take place among the previously identified genes. In order to cope with the large amount of false positive microRNA predicted targets, usually present in public repositories, we only collected microRNA targets confirmed by miRTarBase [24]. MiRTarBase collects, in fact, only experimentally validated microRNA-target interactions, which resulted in a smaller and more reliable set of microRNAs. Transcription factors have been collected from TargetMine [25].

The enhanced network is depicted in Figure 5 and the list of enhanced entities (transcription factors and microRNAs) is synoptically reported in Table 3.

We validated through literature the whole set of discovered regulatory entities, to estimate both their role under shear stress conditions and the overall reliability of the resulting enhanced network. Results confirmed the expected reliability of the enhanced network, given an overall validation of 88,3% (40 out 46 microRNAs and 13 out of 14 transcription factors appear directly involved in shear stress or in shear stress induced inflammation).

Overall, the proposed enhanced network confirms the role of CXCR4, TNFAIP3, and CASP8 plus X-box Binding Protein 1 (XBP1) in shear stress, further enlarging the set of regulations and highlighting the presence of common regulatory motif composed by TFs and microRNAs, already recognized in shear stress, that possibly finely tune the pathway behavior.

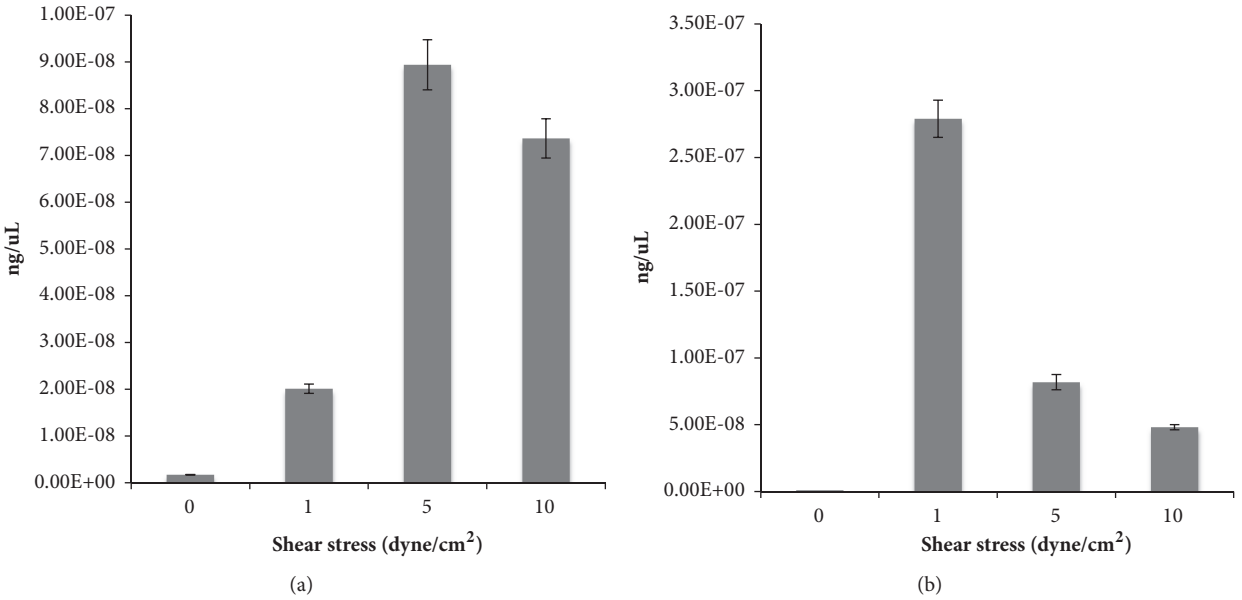

Figure 4: RT-PCR of ICAM-1 (a) and CXCR4 (b) genes in the different shear stress conditions.

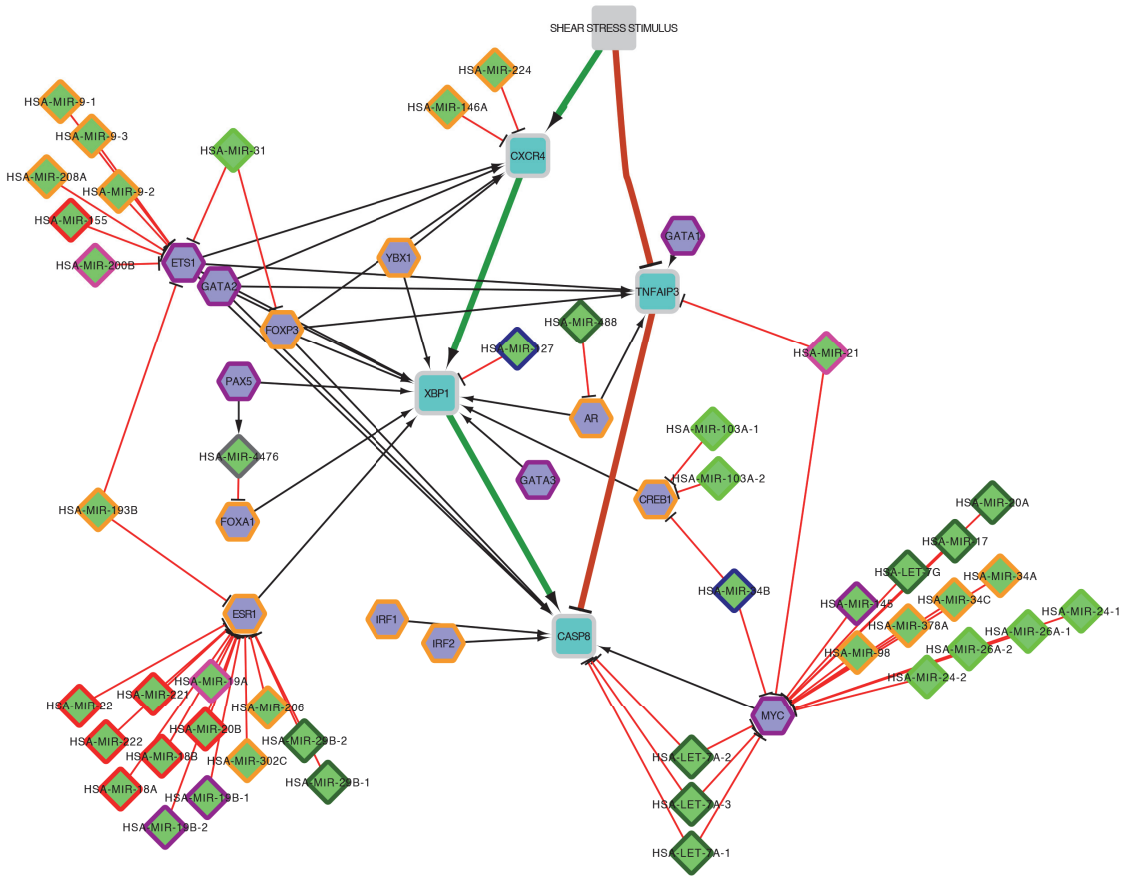

Figure 5: Schematic representation of the enhanced network. (i) Squares represent the genes previously identified in wet lab assessment, (ii) diamonds represent microRNAs, and (iii) hexagons represent transcription factors. The node stroke represents the role of each regulator as follows: angiogenesis (red), apoptosis (dark green), atherogenesis (light green), Inflammatory response (orange), NO synthesis and response (pink), senescence (blue), shear stress regulation (purple), and previously identified genes (light gray).

Table 3: List of microRNA and transcription factors added to the regulatory network after the enhancing process. Node represents the regulator's name, type describes if the regulator is a transcription factor or a microRNA, PMID is the PubMed ID of the reference paper, and Function contains the overall regulatory function.

Node	Type	PMID	Function
HSA-MIR-155	External Mirna	23512606	Angiogenesis
HSA-MIR-18A	External Mirna	21171923	Angiogenesis
HSA-MIR-18B	External Mirna	21171923	Angiogenesis
HSA-MIR-20B	External Mirna	24158362	Angiogenesis
HSA-MIR-22	External Mirna	23512606	Angiogenesis
HSA-MIR-221	External Mirna	23512606	Angiogenesis
HSA-MIR-222	External Mirna	23512606	Angiogenesis
HSA-LET-7A-1	External Mirna	21171923	Apoptosis
HSA-LET-7A-2	External Mirna	21171923	Apoptosis
HSA-LET-7A-3	External Mirna	21171923	Apoptosis
HSA-LET-7G	External Mirna	21171923	Apoptosis
HSA-MIR-17	External Mirna	21171923	Apoptosis
HSA-MIR-20A	External Mirna	21171923	Apoptosis
HSA-MIR-29B-1	External Mirna	21171923	Apoptosis
HSA-MIR-29B-2	External Mirna	21171923	Apoptosis
HSA-MIR-488	External Mirna	21710544	Apoptosis
HSA-MIR-103A-1	External Mirna	22267480	Atherogenesis
HSA-MIR-103A-2	External Mirna	22267480	Atherogenesis
HSA-MIR-24-1	External Mirna	23729638	Atherogenesis
HSA-MIR-24-2	External Mirna	23729638	Atherogenesis
HSA-MIR-26A-1	External Mirna	22267480	Atherogenesis
HSA-MIR-26A-2	External Mirna	22267480	Atherogenesis
HSA-MIR-31	External Mirna	23729638	Atherogenesis
HSA-MIR-146A	External Mirna	21171923	Inflammatory response
HSA-MIR-193B	External Mirna	23729638	Inflammatory response
HSA-MIR-206	External Mirna	23381794	Inflammatory response
HSA-MIR-208A	External Mirna	23797034	Inflammatory response
HSA-MIR-224	External Mirna	22178270	Inflammatory response
HSA-MIR-302C	External Mirna	21490602	Inflammatory response
HSA-MIR-34A	External Mirna	21171923	Inflammatory response
HSA-MIR-34C	External Mirna	26779287	Inflammatory response
HSA-MIR-378A	External Mirna	25104350	Inflammatory response
HSA-MIR-9-1	External Mirna	23797034	Inflammatory response
HSA-MIR-9-2	External Mirna	23797034	Inflammatory response
HSA-MIR-9-3	External Mirna	23797034	Inflammatory response
HSA-MIR-98	External Mirna	25623956	Inflammatory response
HSA-MIR-19A	External Mirna	22047531	NO synthesis and response
HSA-MIR-200B	External Mirna	23975169	NO synthesis and response
HSA-MIR-21	External Mirna	22047531	NO synthesis and response
HSA-MIR-127	External Mirna	24282530	Senescence
HSA-MIR-34B	External Mirna	20424141	Senescence
HSA-MIR-145	External Mirna	23512606	Shear stress regulation
HSA-MIR-19B-1	External Mirna	25012134	Shear stress regulation
HSA-MIR-19B-2	External Mirna	25012134	Shear stress regulation
AR	Transcription Factor	26097556	Inflammatory response
CREB1	Transcription Factor	25883219	Inflammatory response
ESR1	Transcription Factor	26808832	Inflammatory response
FOXA1	Transcription Factor	24801505	Inflammatory response
FOXP3	Transcription Factor	21873519	Inflammatory response

Table 3: Continued.

Node	Type	PMID	Function
IRF1	Transcription Factor	23934855	Inflammatory response
IRF2	Transcription Factor	2475256	Inflammatory response
YBX1	Transcription Factor	23872051	Inflammatory response
ETS1	Transcription Factor	18636553	Shear stress regulation
GATA1	Transcription Factor	15231498	Shear stress regulation
GATA2	Transcription Factor	15231498	Shear stress regulation
GATA3	Transcription Factor	15231498	Shear stress regulation
MYC	Transcription Factor	9598824	Shear stress regulation
PAX5	Transcription Factor	19616560	Shear stress regulation

4. Discussion

In this work, our attention was focused on first modifications occurring to endothelial cell gene profile, able to induce the development of an atherosclerotic lesion. The analysis of results shows a peculiar pattern of gene modulation linked to inflammation and we highlight a possible gene network involved in this pathological process.

The presence of low shear or nonlaminar flow is able to induce changes in gene expression profile that predispose endothelium to the initiation and development of atherosclerotic lesions [26, 27].

We decided to submit HUVECs at a well-defined range of shear stress values (0, 1, 5, and 10 dyne/cm^2) to cover a wide spectrum of conditions, according to flow ranges employed in the majority of publications [28–31]. Physiologic fluid flows in the ranges of about 1-50 dyne/cm^2 [32, 33] are found in vivo in the majority of blood vessels and its disturbance can produce pathogenic states such as thrombosis and atherosclerosis. It has verified that regions of arterial circulation prone to develop atherosclerosis are characterized by "disturbed," oscillatory flow with low levels of shear stress (mean time-averaged shear stress $\leq$ 1 dyne/cm^2) [34]. The range of flow we chose let us analyze physiological shear stress (5 and 10 dyne/cm^2) with respect to a proatherogenic shear rate (1 dyne/cm^2).

Obtained results show that shear stress modulates cell structure in terms of cytoskeletal reorganization with a cobblestone architecture at all shear stress values and with a smooth elongation at 10 dyne/cm^2. This behavior is similar to that observed by Sakamoto [35], where only cell exposed to high shear stress showed a tendency to align parallel to the direction of flow. Our results are similar to those from literature also in terms of cell viability: a significant increase of cell metabolic activity was obtained at low shear stress, as previously observed by Conway et al. [31].

Furthermore, the comparison of different shear rates on a uniform culture of human endothelial cells by microarray analysis highlights the earliest potential modulator of the disease process.

In our genetic study, Affymetrix analysis showed a regulation of various genes, in particular linked to inflammation and apoptosis, such as CXCR4, ICAM-1, Caspase-8, and TNFAIP3 genes. These experiments highlighted a strong upregulation of CXCR4 and a >2 fold increase for Caspase-8; on the other hand, remarkable downregulation of TNFAIP3 and, less in amplitude, of ICAM-1 was evidenced. Furthermore, the expression values of two genes, CXCR4, for its highest overexpression, and ICAM-1, known to be involved in the atherosclerotic process, were confirmed with RT-PCR.

The overexpression of CXCR4 seems to indicate an active state of endothelial cells subject to a low shear stress, a first signal of "reaction" to the atheroprone waveform of flow in the bioreactor. CXCR4, as chemokine receptor, is involved in the MIF action. This is widely expressed in macrophages and endothelial cells of atherosclerotic plaques and induces integrin-independent arrest and transmigration of monocytes and T cells. MIF contributes, in this way, to lesion progression and plaque inflammation trough the interaction with CXCR4 [12, 13, 36].

The MIF involvement in atherosclerotic plaque progression was confirmed using MIF$^{-/-}$/LDL-R$^{-/-}$mice on an atherogenic diet for 12 and 26 weeks [37]. Interestingly, in vitro adhesion assays revealed that monocyte adhesion on ox-LDL-treated human aortic endothelial cells depends almost completely on endothelial MIF and stimulation of aortic endothelial cells with MIF for 2 h induced monocyte adhesion under flow conditions, providing preliminary hints to a chemokine-like function of MIF in leukocyte recruitment [38].

Regarding ICAM-1 gene levels, our finding shows a decrease of ICAM-1 gene expression at low shear stress, differing from previously published data [28]. These conflicting results can be explained both by the absence of an inflammatory stimuli [14, 15] and by its role in elaboration of "mature" atherosclerotic lesions [39]. On the other hand, Conway et al. [31], comparing the effects of reversing shear stress (time-average: 1 dyne/cm^2, max: +11 dyne/cm^2, min: -11 dyne/cm^2, 1 Hz), arterial steady shear stress (15 dyne/cm^2), and low steady shear stress (1 dyne/cm^2) on surface expression of ICAM-1, cell proliferation, and monocyte adhesiveness, verified that only reversing shear stress exposure induced monocyte adhesion. ICAM-1 expression was also lower at 1 dyne/cm^2 with respect to 15 dyne/cm^2, confirming our results. Laminar flow produced a significant time-dependent increase in the surface expression of ICAM-1 on endothelial cells, while

turbulent flow did not affect its surface expression [40, 41]. A direct link between shear stress and ICAM-1 gene expression, through the identification of a Shear Stress Response Element (SSRE) in the promoter region for the ICAM-1 gene, has been established by Resnick [42].

Considering the literature, also Caspase-8 and TNFAIP3, the other two genes most modulated in our experiments, can assume an interesting role.

Caspase-8 is an initiator of the extrinsic pathway of apoptosis. Scharner [43] demonstrated the strictly link between Caspase-8 and CXCR4. Aging with a blocker of apoptotic agent, a downregulation of CXRC4 expression on EPCs in vitro was observed. On the other hand, the role of altered shear stress, like low flow, as a regulator of cell apoptosis has recently been demonstrated by Li and Xu [44]. In endothelial cells, disturbed flow stress resulted in a sustained activation of beta-1 integrin system linked to the X-box Binding Protein 1 (XBP1), which functions as a key signal transducer resulting in increase of caspase activation in endothelial cells [45]. Referring to the literature, in our work a possible correlation between CXCR4 and Caspase-8 can be shown, furnishing an interesting new research starting point

Regarding TNFAIP3, in our experimental model, low flow downexpressed the gene, which has normally a protective function against apoptosis and inflammation through the blockade of the transcription factor, Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-kB) [46]. In cultures derived from primary cells, various investigators have found that overexpression of TNFAIP3 protects cells from cycloheximide/TNF–, ceramide/TNF–, and IL-1–mediated apoptosis [47–49]. This link between apoptosis and TNFAIP3 was also demonstrated by Daniel et al. [50]. TNFAIP3 also blunts natural killer cell-mediated EC apoptosis by inhibiting Caspase-8 activation. These data show the cytoprotective effect of TNFAIP3 in ECs. In our experiments the downregulation of TNFAIP3 may explain the correlated upregulation of Caspase-8 gene, favoring an inflammatory and proatherogenic cell condition.

The wet lab results were further exploited resorting these by bioinformatics analysis: the set of previous identified genes (i.e., CXCR4, TNFAIP3, and CASP8 plus XBP1) was enriched with the set of its upstream regulators (i.e., microRNAs and transcription factors) (Figure 5). This approach allowed deeper insight about the regulatory gene dynamics under low shear stress conditions.

The enhancing process added to the network 59 new interactors (14 transcription factors and 45 microRNAs) (Table 3), each of them has been validated in literature in order to evaluate its role and to measure the overall reliability of the enhanced network. Interestingly, all the newly introduced regulators, except one, appear strongly related to the shear stress phenomenon.

The 14 transcription factors appear evenly distributed between inflammatory response (8 out of 14: IRF2, FOXP3, YBX1, IRF1, FOXA1, CREB1, AR, and ESR1) and shear stress regulation (6 out of 14: MYC, GATA1, GATA2, GATA3, ETS1, and PAX5). In particular, ETS1, GATA2, and FOXP3 act as hubs by targeting all the previously identified genes and ESR1, MYC, and ETS1 are, in turn, coregulated by large separated groups of microRNAs each.

The 44 validated microRNAs are distributed among multiple classes reported as commonly involved in shear stress phenomenon: 13 microRNAs are involved in inflammatory response, 9 in apoptosis, 7 in both angiogenesis and atherogenesis, 3 in both NO synthesis and response and shear stress regulation, and finally, 2 in senescence.

Three large heterogeneous separated groups of microRNAs appear to act in concert to respectively regulate: ETS1 (hsa-mir-9-1, hsa-mir-9-2, hsa-mir-9-3, hsa-mir-155, hsa-mir-208a, hsa-mir-200b), ESR1 (hsa-mir-18a, hsa-mir-18b, hsa-mir-19a, hsa-mir-19b-1, hsa-mir-19b-2, hsa-mir-206, hsa-mir-20b, hsa-mir-22, hsa-mir-221, hsa-mir-222, hsa-mir-29b-1, hsa-mir-29b-2, hsa-mir-302c), and MYC (hsa-let-7g, hsa-mir-145, hsa-mir-17, hsa-mir-20a, hsa-mir-24-1, hsa-mir-24-2, hsa-mir-26a-1, hsa-mir-26a-2, hsa-mir-34a, hsa-mir-34c, hsa-mir-378a).

7 microRNAs (hsa-let-7a-1, hsa-let-7a-2, hsa-let-7a-3, hsa-mir-193b, hsa-mir-21, hsa-mir-31, hsa-mir-34b) act as regulatory bridges among highly connected regulatory clusters by regulating couple of targets each (transcription factors and genes).

The only unvalidated element is hsa-mir-4476; nevertheless such microRNA has been recently discovered and no clues about its role are actually available in literature. Giving the overall reliability of the rest of the enhanced network, the lack of validation of hsa-mir-4476 makes it a good candidate for further studies to elucidate its possible involvement in shear stress.

Furthermore, hsa-mir-4476 is coexpressed by PAX5 and appears participating in incoherent feed-forward loop regulatory motif [51]. Such motif acts as a pulse generator and response accelerator: PAX5 activates XBP1, but also represses XBP1 by the repression of FOXA1 mediated by hsa-mir-4476. As a result, when a signal causes PAX5 to assume its active conformation, XBP1 is rapidly produced. However, after some time, hsa-mir-4476 levels accumulate to reach the repression threshold for the XBP1 promoter. As a result, XBP1 production decreases and its concentration drops, resulting in pulse-like dynamics.

Integrating our data with the state of the art related to genes under investigation, a potential relation between CXCR4, Caspase-8, and TNFAIP3 in the development of the early phases of atherosclerotic disease can be shown (Figure 6).

In fact, endothelial chemokine receptor CXCR4 activation at low shear stress (Figure 6(b)) can play a role in the development of atherosclerosis acting as an important mediator of the endothelial response to damage through interaction with MIF.

The concomitant overexpression of Caspase-8, involved in early phase of apoptotic process, seems closely related to upregulation of CXCR4 and the downregulation of TNFAIP3 suggest that all these gene modulations can synergistically promote an initial inflamed cell phenotype at low flow with a cascade of events that come ahead the endothelial activation through adhesion molecules expression.

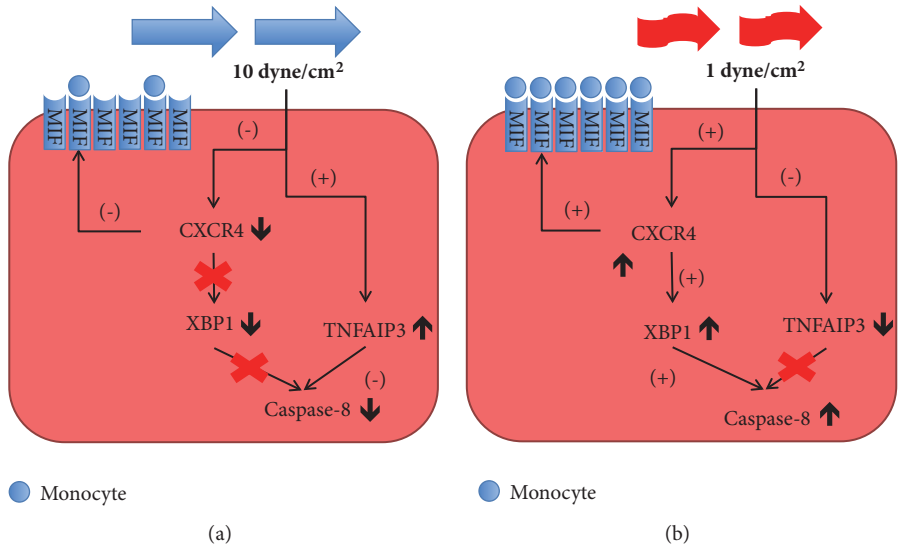

Figure 6: Representation of relations between CXCR4, TNFAIP3, and Caspase-8 in HUVECs under physiological and low shear stress. At physiological level (a), shear stress is able to downregulate CXCR4, limiting its role in XBP-1 stimulation with reduction of apoptotic activity of Caspase-8; in parallel, there is low interaction with MIF, reducing monocyte adhesion and trans-migration in vascular wall. TNFAIP3 is highly expressed and inhibits Caspase-8. This behavior changes when endothelial cells are subjected to low shear stress (b): CXCR4 is overexpressed, promoting its interaction with MIF and consequent increased monocyte adhesion. Furthermore, it stimulates XBP-1, with augmented apoptotic activity not counteracted by TNFAIP3, which is downregulated.

ICAM-1 downregulation, instead, can be justified by its limited role in the very early stages of disease, which become more important in following phases of atherosclerosis [50, 52].

5. Conclusions

Atherosclerotic process is a pathological event where several inputs participate to create a network with several players. In particular, our findings may shed light on specific patterns: the role of specific mediators of chemokine, caspase, and inflammatory systems in situations of low shear stress that might be relevant in the initial phases of atherosclerotic process and for fundamental regulatory action of laminar shear stress in preservation of endothelial cell integrity and survival.

Although our work presents some limitations, like RT-PCR gene analysis focused only on CXCR4 and ICAM-1 and no experiments focused on the possible cause-effect relationship of this hypothesis, it highlights that the concomitant modulation of CXCR4, Caspase-8, ICAM-1, and TNFAIP3 at low shear stress may predispose endothelium to a preinflamed phase.

At the same time, the emerging findings together with an integrated approach by the way of bioinformatics tools, which has enriched the network of specific genes, transcription factors, and microRNAs, improve knowledge about comprehension of chemokine role in early stages of patient plaque development focalizing a chemokine hypothesis-driven to be analyzed with further experiments.

Abbreviations

CASP8:	Caspase-8
Cp:	Crossing point
CXCR4:	Chemokine (C-X-C motif) Receptor 4
DEG:	Different Expressed Genes
ECGM:	Endothelial Cell Growth Medium
FC:	Fold Change
GEO:	Gene Expression Omnibus
HUVECs:	Human Umbilical Vein Endothelial Cells
ICAM-1:	Intercellular Adhesion Molecule 1
MIF:	Macrophage Migration Inhibitory Factor
NF-kB:	Nuclear Factor kappa-Light-Chain-Enhancer of Activated B Cells
Ox-LDL:	Oxidized Low Density Lipoprotein
PBS:	Phosphate Buffered Saline
PDMS:	Polydimethylsiloxane
ReNE:	Regulatory Network Enhancer
RMA:	Robust Multiarray Average
RT-PCR:	Real-Time Polymerase Chain Reaction
TF:	Transcription Factors
TNF-alpha:	Tumor Necrosis Factor alpha
TNFAIP3:	Tumor Necrosis Factor Alpha-Induced Protein 3
XBP-1:	X-box Binding Protein 1.

Data Availability

The microarray dataset supporting the conclusions of this article is available in the NCBI-GEO repository http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE45225.

Ethical Approval

This research was approved by Local Ethics Committee of Area Vasta Nord Ovest. The umbilical cords were stored in PBS at 4°C, sent to our laboratory within 1 hour of delivery and treated anonymously conforming to the principles outlined in the Declaration of Helsinki.

Consent

Fresh umbilical cords were recovered from healthy females at the Obstetrics and Gynecology Unit of Azienda Ospedaliera Universitaria Pisana, after obtaining written informed consent for use of these samples.

Conflicts of Interest

There are no conflicts of interest to declare by all authors.

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Research Article

Bone Marrow-Derived Endothelial Progenitor Cells Contribute to Monocrotaline-Induced Pulmonary Arterial Hypertension in Rats via Inhibition of Store-Operated Ca^{2+} Channels

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Purpose. This study aimed to explore whether bone marrow- (BM-) derived endothelial progenitor cells (EPCs) contributing to monocrotaline- (MCT-) induced pulmonary arterial hypertension (PAH) in rats via modulating store-operated Ca^{2+} channels (SOC). *Methods*. Sprague Dawley (SD) rats were assigned into MCT group (n = 30) and control group (n = 20). Rats in MCT group were subcutaneously administered with 60 mg/kg MCT solution, and rats in control group were injected with equal amount of vehicle. After 3 weeks of treatment, right ventricular systolic pressure (RVSP) and right ventricular hypertrophy index (RVHI) of two groups were measured, and BM-derived EPCs were isolated. Immunochemistry identification and vasculogenesis detection of EPCs were then performed. $[Ca^{2+}]_{cyt}$ measurement was performed to detect store-operated calcium entry (SOCE) in two groups, followed by determination of Orai and canonical transient receptor potential (TRPC) channels expression. *Results*. After 3 weeks of treatment, there were significant increases in RVSP and RVHI in MCT group compared with control group, indicating that MCT successfully induced PAH in rats. Moreover, the SOCE ($[Ca^{2+}]_{cyt}$ rise) in BM-derived EPCs of MCT group was lower than that of control group. Furthermore, the expression levels of Orai3, TRPC1, TRPC3, and TRPC6 in BM-derived EPCs were decreased in MCT group in comparison with control group. *Conclusions*. The SOC activities were inhibited in BM-derived EPCs of MCT-treated rats. These results may be associated with the depressed expression of Orai3, TRPC1, TRPC3, and TRPC6, which are major mediators of SOC.

1. Introduction

Pulmonary arterial hypertension (PAH) is a fatal disorder characterized by an increase in pulmonary vascular resistance [1, 2]. It always leads to right ventricular (RV) failure and death [3, 4]. Despite advances in therapeutic options, this disease represents an incurable disease due to progressive clinical deterioration and an unacceptably high early mortality [5, 6]. Therefore, elucidation of key pathological mechanism underlying PAH development is still imperative.

Accumulating evidences have confirmed that excessive pulmonary vascular remodeling is responsible for the elevated pulmonary vascular resistance in PAH [7–9]. In pulmonary arterial smooth muscle cells (PASMCs), the rise in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) is identified as a key trigger for promoting the proliferation of PASMCs

and pulmonary vasoconstriction, both leading to pulmonary vascular remodeling [10–13]. Moreover, the profound pulmonary vascular remodeling and alterations in Ca^{2+} homeostasis in PASMCs may result in the development of PAH [14]. These findings support the pathogenic role of Ca^{2+} signaling in PAH.

Endothelial progenitor cells (EPCs) are considered to be important in maintaining vascular homeostasis, which can be mobilized from the bone marrow (BM) and resident locally in the lung [15]. EPCs are found to have a key role in the endothelial repair [16, 17]. It is reported that BM-derived EPCs can repair the monocrotaline- (MCT-) damaged lung in the rat MCT model of PAH [18]. Moreover, EPCs can induce neovascularization, suggesting the promising clinical application of the EPCs cell therapy to PAH [19]. However, the possible mechanism of EPCs in regulating pulmonary vascular remodeling during PAH development is largely unknown.

Notably, store-operated Ca^{2+} channels (SOC) is expressed in human EPCs [20]. Given the pathogenic role of Ca^{2+} signaling in PAH, the present study investigated whether BM-derived EPCs contributed to PAH in the MCT rat model via modulating SOC. To study this, we established the MCT rat model that was widely used to investigate PAH in rodents [20–22]. Then BM-derived EPCs were isolated. $[Ca^{2+}]_{cyt}$ measurement was performed to detect store-operated calcium entry (SOCE) in BM-derived EPCs of MCT rat model and controls, followed by determination of SOC regulators, Orai, and canonical transient receptor potential channel (TRPC) expression. Our findings will provide a new insight for better understanding of PAH pathogenesis.

2. Materials and Methods

2.1. Animals and Treatment. A total of 50 male Sprague Dawley (SD) rats (weighing 150-180 g) were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). Rats were divided into MCT group (n = 30) and control group (n = 20). Rats in MCT group were subcutaneously administered with 60 mg/kg MCT solution 25 mg/ml diluted in vehicle (1:4 mixture of dehydrated ethanol-normal saline). Rats in control group were injected with equal amount of vehicle. This study was approved by the institutional ethical committee for animal care and use. During the treatment period, the behavior and general status were observed daily.

2.2. Measurement of Pulmonary Hemodynamic and Right Ventricular Hypertrophy. After 3 weeks of treatment, the rats were intraperitoneally anesthetized with 35mg/kg pentobarbital sodium (Abbott Laboratories, Montreal, Canada). Right ventricular systolic pressure (RVSP) of rats in each group was measured by inserting a Millar catheter (Millar, Inc., TX, USA) into RV. Moreover, the RV was separated from the left ventricle (LV) and septum (S) for further detection of RV hypertrophy. The right heart hypertrophy index (RVHI) [RV/(LV+S)] was calculated as the ratio of RV weight to (LV+S) weight.

2.3. Isolation of Rat BM-Derived EPCs. Rats in each group were sacrificed by exsanguination, and BM was then aspirated from bilateral femurs and tibias of rats aseptically. Using density gradient centrifugation Histopaque®-1083 solution (Sigma-Aldrich, MS, USA), mononuclear cells (MNCs) were isolated from BM. To produce EPCs, BM-isolated MNCs were then resuspended in EGM-2 MV medium (Lonza, MD, USA), seeded into fibronectin (5 $\mu g/cm^2$) coated six-well plates at a density of $3 \times 10^6/cm^2$ and maintained at a 37°C incubator for 8 days. EGM-2 MV medium was replaced every two days.

2.4. Immunochemistry Identification. After 8 days of incubation, the fibronectin-adherent EPCs were identified by incubation with 10 μg/ml of fluorescently labeled acetylated-low-density lipoprotein (Dil-ac-LDL; Molecular Probes, Eugene, OR, USA) overnight and 10 μg/ml of fluorescently FITC-labeled *Ulex europaeus* agglutinin 1 (UEA-1; Sigma-Aldrich, MS, USA) for 4 h at room temperature using an immunochemistry method [4, 23]. The images were captured by Leica-SP5 confocal microscopy (Leica, Germany), and both FITC-UEA-1 and Dil-ac-LDL positive cells were considered as EPCs.

2.5. Detection of Vasculogenesis. To mimic vasculogenesis of EPCs, the vascular network formation was observed. Briefly, 24-well plates were presolidified Matrigel (BD Biosciences, MA, USA) for 30 minutes. EPCs were seeded into 24-well plates containing 500 μl EGM-2-MV medium at a density of 7.5×10^4 cells/$2cm^2$ Matrigel. After incubation for 5 h, the developing vascular network in 10 fields was observed under a microscope (Nikon, Japan). The length of vascular network per field was calculated.

2.6. $[Ca^{2+}]_{cyt}$ Measurement. According to the protocols described previously [24, 25], $[Ca^{2+}]_{cyt}$, defined as the ratio of fluorescence intensities of 340 to 380 nm wavelengths (F340/F380), was monitored using fura-2 acetoxymethyl ester (Invitrogen-Molecular Probes, Eugene, OR) and then imaged with NIS Elements 3.2 software (Nikon).

To determine whether the different amplitude of $[Ca^{2+}]_{cyt}$ increase between MCT and control groups was caused by SOCE, 10 μM CPA was extracellularly applied, which is a sarco/endoplasmic reticulum Ca^{2+}-ATPase inhibitor that induces Ca^{2+} influx. The $[Ca^{2+}]_{cyt}$ rise (ΔRatio) of MCT and control groups was then detected.

2.7. Real-Time PCR. Total RNA was extracted from EPCs in MCT and control groups using TRIzol® reagent (Invitrogen, Burlington, ON, Canada). The quality and concentration of total RNA were then determined with a spectrophotometer (NanoDrop 2000, Thermo Scientific, USA). Reverse transcription into cDNA was conducted using the PrimeScriptTM RT Master Mix Kit (Takara, Japan). The expression levels of Orai and TRPC channel were then detected by real-time PCR on the Applied Biosystems Real-Time PCR System 7500 Fast (Applied Biosystems, Foster City, CA, USA). The primers (forward and reverse, 5'- 3') for amplification of

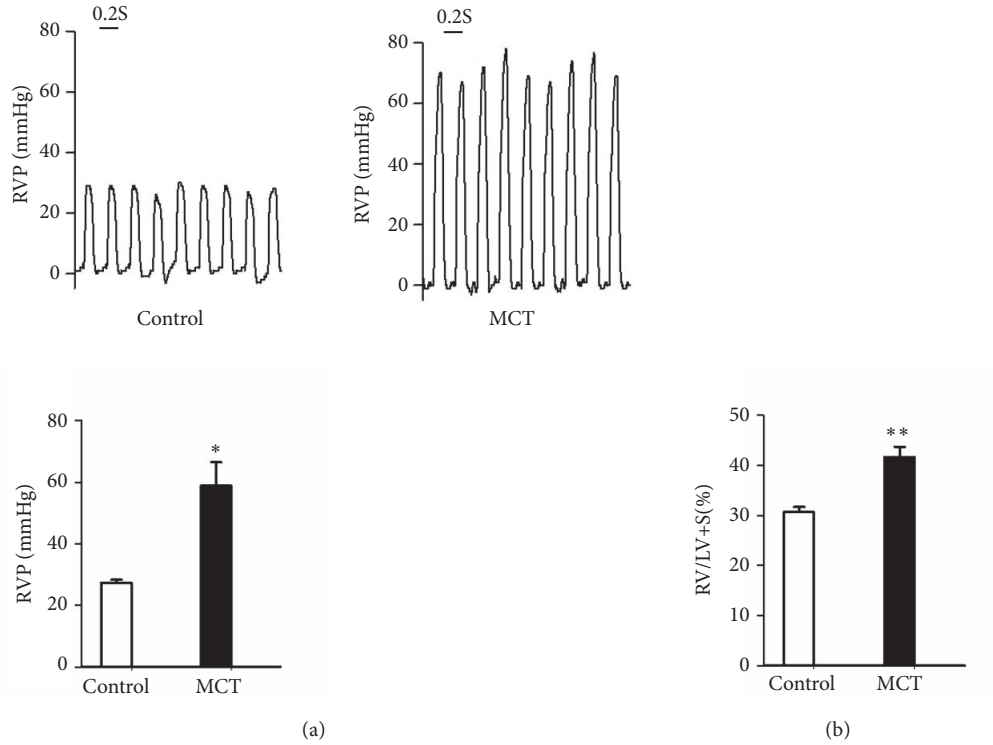

Figure 1: Monocrotaline (MCT) induced pulmonary arterial hypertension (PAH) in rats after 4 weeks of MCT treatment. (a) RVSP of rats in each group. (b) The RVHI of rats in each group. RVSP: right ventricular systolic pressure; RVHI: right ventricular hypertrophy index. $*$ $P < 0.05$ and $**$ $P < 0.01$ compared with control group.

genes were as follows: Orai 1: AAGTTCTTACCGCTCAAGAGGCAG and AGCGGTAGAAGTGAACGGCAAAGA; Orai 2: TGTGGGTCTCATCTTCGTGGTCTT and TGAGCTTGTGCAGTTCCTCGATCT; Orai 3: TAGTGCCTGCACCACTGTGTTAGT and ATTGTGGATGTTGCTCACGGCTTC; TRPC7: AGACACGGAAGAGGTGGAAGCAAT and AGTTAGGGTGGGCAACGAACTTCT; TRPC6: TCTGGCTGCTCATTGCCAGGAATA and AGAGTGGCTGAAGGAGTCATGCTT; TRPC5: AGTTCACACCAGACATCACACCCA and TGAACTGGACACACACTCCACACA; TRPC4: AGTTTATCTGCCACACAGCCTCCT and AGTCCGCCATCCCACATCTGTTTA; TRPC3: TCTTCCTGGGTCTGCTTGTGTTCA and TGTCCATGTGAACTGGGTGGTCTT; TRPC2: TCCTGTGAAGATCAGCCATGTGGT and TGTCTGGGTTCAGCAAGTTCTCCA; and TRPC1: ACAGAAGATGCAGAGCACAGACCA and AAGTCCGAAAGCCAAGCAAATCCC. Each sample was analyzed in triplicate. Cycling parameters were set as follows: 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s.

2.8. Statistical Analysis. All experiments were independently repeated three times. All measurement data were presented as the mean ± standard deviation (SD) and analyzed for significant difference by using an unpaired Student's *t*-test Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA). A value of $P < 0.05$ indicated a statistically significant result.

3. Results

3.1. The General Status of Animals. During the animal experiment, 2 and 3 rats in MCT group died in the third and fourth weeks after MCT injection, respectively. As a result, 45 rats were enrolled in this study. After 3 weeks of treatment, rats in MCT group appeared to obviously have asarcia, dyspnea, and chest and ascites formation, together with liver congestion and swelling, heart enlargement, right ventricular hypertrophy, and other heart failure manifestations. Rats in control group did not exhibit any abnormities.

3.2. MCT-Induced PAH in Rats. MCT was used to induce PAH in rats in this study. The results showed that rats in MCT group developed PAH after 3 weeks of MCT treatment as reflected by a remarkable increase in RVSP: 59.40 ± 8.13 mmHg in MCT rats versus 27.45 ± 0.89 mmHg in control rats ($P < 0.05$, Figure 1(a)). Moreover, the RVHI in MCT group (41.69 ± 2.00%) was significantly increased compared with that in the control group (31.00 ± 1.00%) ($P < 0.01$, Figure 1(b)). These data indicated that MCT successfully induced PAH in rats.

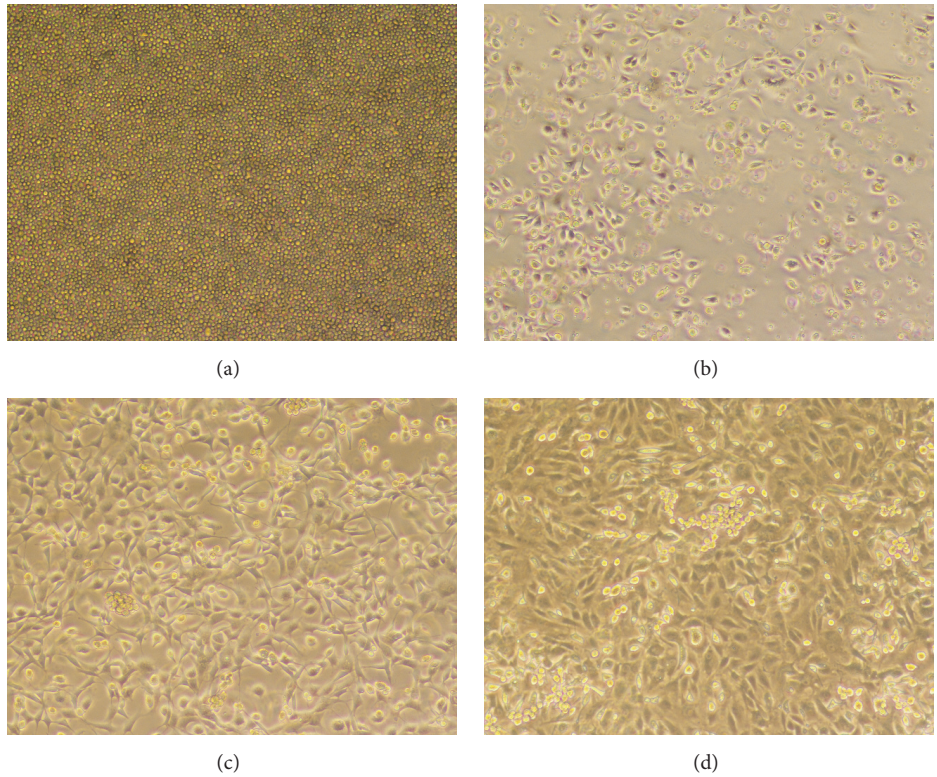

Figure 2: The morphological changes of bone marrow- (BM-) derived endothelial progenitor cells (EPCs) after culture for 1 (a), 4 (b), 6 (c), and 8 (d) days in the selective medium.

3.3. Identification of BM-Derived EPCs. The morphological changes of EPCs were observed after culture for 1, 4, 6, and 8 days in the selective medium. After 1 day of culture, cells were adhered to the wall, but their morphology was not uniform and most of these cells were round (Figure 2(a)); some cells were polygonal or spindle-shaped after 4 days (Figure 2(b)); the majority of cells were polygonal or spindle-shaped after 6 days (Figure 2(c)); spindle-shaped or polygonal cells showed dominant growth after 8 days, paving stone-like arrangement and clear cell gaps (Figure 2(d)).

Furthermore, immunochemistry identification was performed after culture for 8 days in the selective medium. Double stained with FITC-UEA-1 and Dil-ac-LDL, EPCs in the adherent MNCs were identified (Figure 3(a)). Moreover, the results showed that 84.40 ± 8.06% of adherent MNCs were identified as double-positive EPCs, confirming that highly purified BM-derived EPCs were successfully isolated.

3.4. Functional Analysis of BM-Derived EPCs by Detection of Vasculogenesis. To further confirm the function of BM-derived EPCs, the vasculogenic potential of EPCs was detected by the vascular network formation test. EPCs were seeded onto the Matrigel for incubation for 5 h. The results showed that the average length of vascular network per field of view was 9.78 ± 0.67 mm (Figure 3(b)).

3.5. MCT Decreased CPA-Induced SOCE in BM-Derived EPCs. To determine whether MCT could regulate SOC in BM-derived EPCs, 10 μM CPA was extracellularly applied to detect the effects of MCT on SOCE. As shown in Figure 4, the $[Ca^{2+}]_{cyt}$ rise (ΔRatio) of MCT group (0.60±0.21) was significantly lower than that in control group (0.91±0.23) ($P < 0.01$), indicating that MCT decreased CPA-induced SOCE.

3.6. The Effects of MCT on the Expression of Orai and TRPC Channel. To further investigate the regulatory mechanism of MCT on SOC, we detected the Orai and TRPC channel expressions, including Orai1-3 and TRPC1-7. In comparison with control group, the expression levels of Orai3, TRPC1, TRPC3, and TRPC6 in BM-derived EPCs were significantly downregulated in MCT group (all $P < 0.05$, Figure 5), indicating that MCT decreased SOCE possible via decreasing the expression of these channel molecules.

4. Discussion

PAH is a degenerating and devastating disease with limited treatment options [26]. Elucidation of the key mechanism underlying PAH will facilitate the development of effective therapeutic strategy for this disease. In this study, the MCT rat model of PAH was successfully established as reflected by a remarkable increase in RVSP and RVHI. Moreover, the delightful results were obtained that the SOCE ($[Ca^{2+}]_{cyt}$) rise in BM-derived EPCs of MCT rat model was significantly inhibited. Furthermore, the expression levels of Orai3,

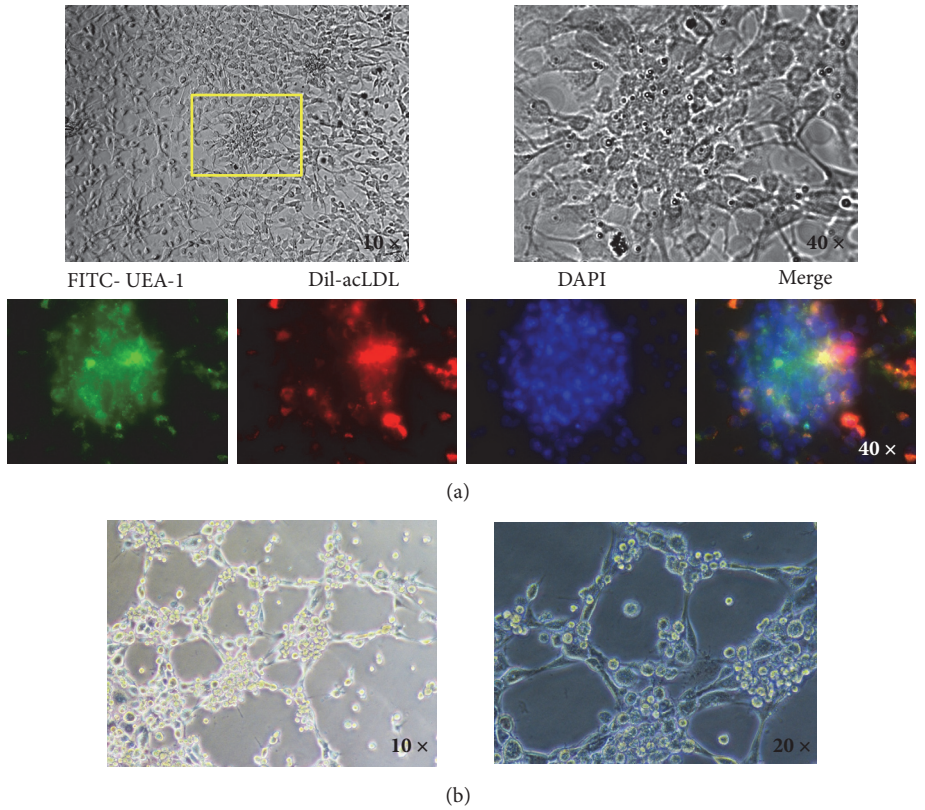

Figure 3: BM-derived EPCs identification with immunochemistry (a), as well as the vascular network formation test (b).

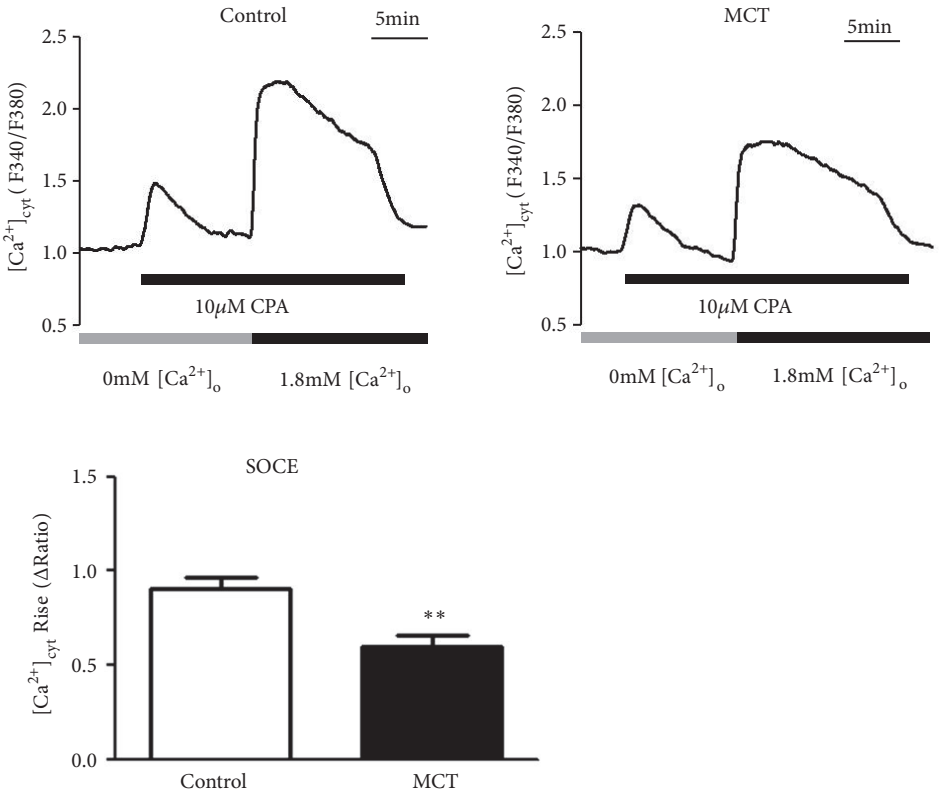

Figure 4: The $[Ca^{2+}]_{cyt}$ rise (SOCE) of MCT and control group. ** $P < 0.01$ compared with control group.

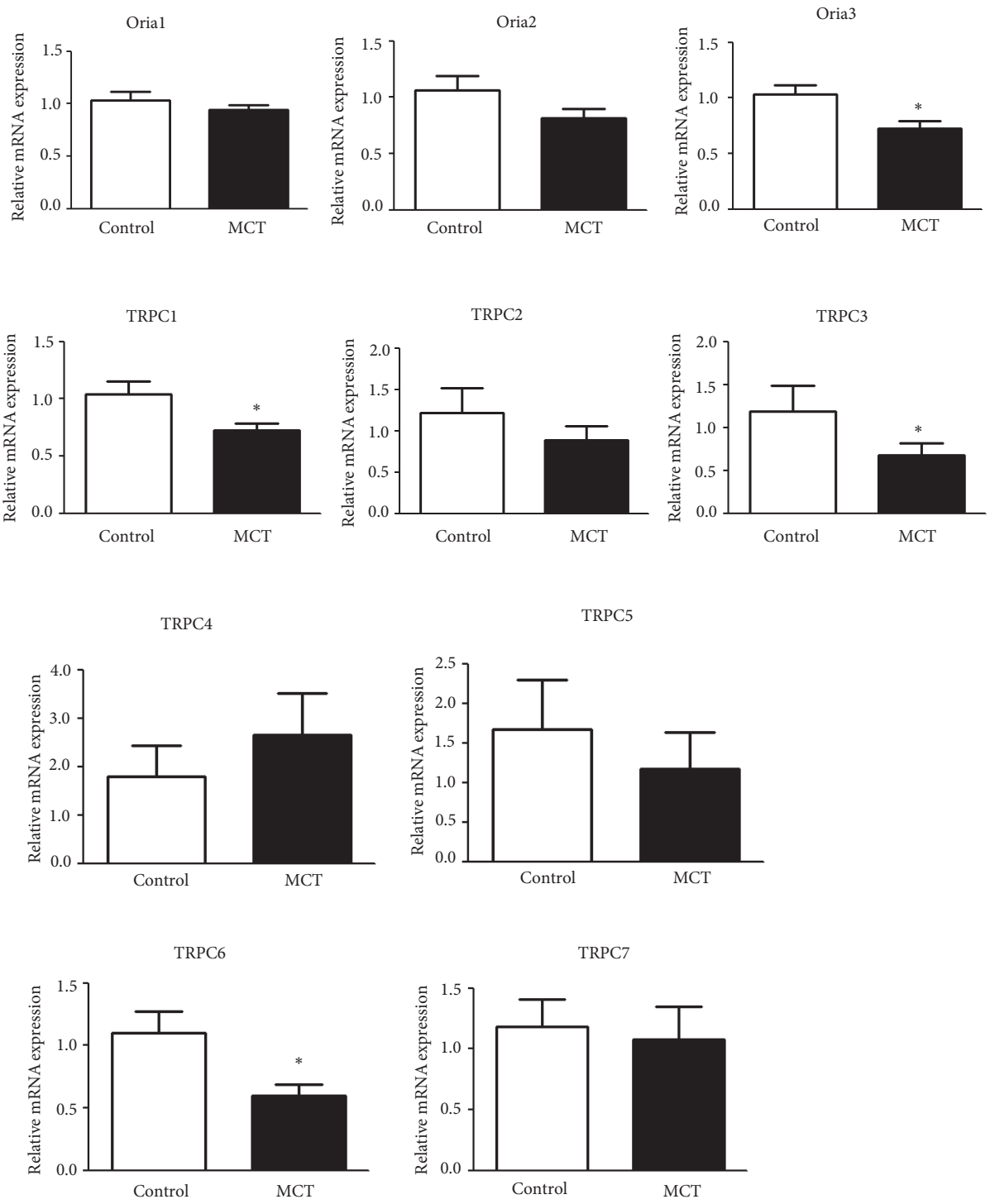

Figure 5: The expression of Orai and TRPC channels, including Orai1, Orai2, Orai3, TRPC1, TRPC2, TRPC3, TRPC4, TRPC5, TRPC6, and TRPC7, in MCT and control group. $* P < 0.05$ compared with control group.

TRPC1, TRPC3, and TRPC6 were markedly decreased in BM-derived EPCs of MCT rat model. These data imply that BM-derived EPCs could be involved in MCT-induced PAH in rats via inhibiting SOCE and related channel expression.

Intracellular Ca^{2+} signaling, as an important second messenger for cell proliferation, is found to play an important role in numerous physiological and pathophysiological processes in PASMCs, like proliferation and hypertrophy [27]. SOCE is a ubiquitous Ca^{2+} entry pathway that is involved in the control of various physiological functions in various cell types [28]. When intracellular Ca^{2+} stores are depleted, SOC can mediate Ca^{2+} influx and increase $[Ca^{2+}]_{cyt}$ [29]. Moreover,

TRPC-dependent SOC is confirmed as an important pathway to mediate the development of MCT-induced PAH [14]. Lin et al. revealed that the enhanced SOCE is responsible for the chronic hypoxia-induced pulmonary hypertension in rats [30]. Zhou et al. demonstrated that SOC regulated endothelial hyperpermeability in severe PAH [31]. These data confirm the pathological role of SOC in PASMCs during PAH development. However, SOC may play a dual role in different cell types. A previous study has shown that SOCE is an important factor in regulating the functions of EPCs and the SOCE inhibition reduces the proliferation and migration of EPCs during atherosclerosis [32]. Wang et al. indicated that SOC inhibition could prevent H_2O_2-induced apoptosis, thus exerting a protective effect on EPCs [33]. Lodola et al. demonstrated that SOC was remodeled and subsequently regulated in vitro angiogenesis in EPCs isolated from tumoral patients [34]. In our study, SOC was inhibited by MCT in BM-derived EPCs of MCT-treated rats. Given the key role of SOC in EPCs, we speculate that BM-derived EPCs may prevent MCT-induced PAH in rats possible via activation of SOC.

Furthermore, both Orai and TRPC proteins are proposed to form SOC [35]. Increasing evidence has suggested the important roles of Orai and TRPC channels in PAH. Orai1, Orai12, and Orai3 can promote SOCE in PASMCs and may serve as potential therapeutic targets for chronic hypoxia-induced pulmonary hypertension [36]. Dragoni et al. suggested that Orai3 is overexpressed in primary myelofibrosis-endothelial colony forming cells (ECFCs) that are EPC subset and thus resulted in the upregulation of SOCE [37]. In this study, Orai3 expression was markedly decreased in BM-derived EPCs of MCT rat model. Considering the key role SOC in PAH, we speculate that inhibition of SOCE due to the downregulation of Orai3 in BM-derived EPCs may play a key role in MCT-induced PAH. In addition, TRPC1 is a major constituent of SOCE and overexpression of TRPC1 can promote SOCE-induced vasoconstriction in rat pulmonary artery [38]. TRPC1 deficiency is found to impair the functions of EPCs on regulating angiogenesis [39]. Moreover, TRPC3 channels are found to be involved in the development of hypertension and its related complications [40]. Poteser et al. indicated that TRPC3 could regulate Ca^{2+} signaling in somatic EPCs [41]. TRPC3-mediated Ca^{2+} signaling in ECFCs is developed as a promising strategy for improving therapeutic angiogenesis in failing hearts [42]. Furthermore, TRPC6 is shown to be critically involved in the disease states of pulmonary vasculature [43]. Yu et al. revealed that a unique genetic variant of the TRPC6 gene promoter might result in pulmonary vascular abnormality in idiopathic PAH by linking abnormal TRPC6 transcription to nuclear factor-κB activity [44]. Additionally, functional interaction between TRPC1 and TRPC6 can mediate Ca^{2+} entry in endothelial cells to promote lung vascular permeability [45]. Blockade of TRPC3 and TRPC6 could be a promising therapeutic strategy for PAH treatment [46]. In this study, the expression of the store-operated TRPC1, TRPC3, and TRPC6 channels was decreased in BM-derived EPCs of MCT rat model, suggesting that BM-derived EPCs may be implicated in MCT-induced PAH via decreasing the expression of these channel molecules.

In conclusion, The SOC activities were inhibited in BM-derived EPCs of MCT-treated rats. These results may be associated with and the depressed expression of Orai3, TRPC1, TRPC3, and TRPC6, which are major mediators of SOC. Our findings may provide a physiological basis for the potential clinical application of the EPCs cell therapy to PAH.

Data Availability

The data used to support the findings of this study are included within the article.

Additional Points

Highlights. (1) MCT-induced PAH in rats successfully. (2) SOCE was decreased by MCT in BM-derived EPCs of MCT-treated rats. (3) The expressions of Orai3 and TRPC1, 3 and 6, were decreased in EPCs of MCT rats.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Ran Miao and Jun Wan contributed equally to this work.

Acknowledgments

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Review Article

The Human Carbonic Anhydrase II in Platelets: An Underestimated Field of Its Activity

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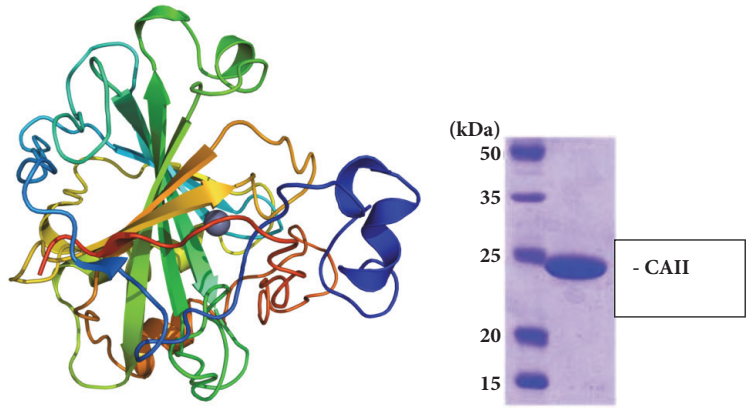
Carbonic anhydrases constitute a group of enzymes that catalyse reversible hydration of carbon dioxide leading to the formation of bicarbonate and proton. The platelet carbonic anhydrase II (CAII) was described for the first time in the '80s of the last century. Nevertheless, its direct role in platelet physiology and pathology still remains poorly understood. The modulation of platelet CAII action as a therapeutic approach holds promise as a novel strategy to reduce the impact of cardiovascular diseases. This short review paper summarises the current knowledge regarding the role of human CAII in regulating platelet function. The potential future directions considering this enzyme as a potential drug target and important pathophysiological chain in platelet-related disorders are described.

1. Carbonic Anhydrases

Carbonic anhydrases constitute a group of zinc containing lyases, classified, according to the Enzyme Catalogue to EC 4.2.1.1., into the lyases subclass "carbon-oxygen lyases" and subclasses "hydrolyses" [1]. They catalyse reversible hydration of carbon dioxide to form bicarbonate ion and proton. Carbonic anhydrase is present in prokaryotic and eukaryotic cells. Genetically they belong to seven subgroups (α, β, γ, δ, ζ, η, θ) that are not evolutionarily related [2–4]. There are 15 carbonic anhydrase isoforms in humans (belonging to the α subgroup) [5]. They occur in various tissues in the cytoplasm, cell membrane, and mitochondria, or as extracellular enzymes (e.g., breast milk) [6]. Carbonic anhydrases (CAs) are almost ubiquitously present in human cells. The members of the CAs family play a role in pH regulation, gas exchange, and ion transport, as well as urine acidification, cerebrospinal fluid secretion, ocular fluid production, bone resorption, fatty acid metabolism, testicular fluid production, and many others [7].

Numerous diseases result from inappropriate function of carbonic anhydrases. There are scarcely several case reports regarding CAs deficiency; nonetheless they constitute a valuable source of knowledge. The erythrocyte CAI deficiency has no clinically relevant consequences [8], but CAII deficiency usually results in osteoporosis, renal tubular acidosis, and brain calcification [9, 10]. CAIII deficiency in skeletal muscles seems to play an import role in pathogenesis of myasthenia gravis [11, 12]. The carbonic anhydrase VA deficiency may present as early-onset liver failure with hyperammonemia, hyperlactatemia, and ketonuria [13]. CAXII deficiency may promote hyponatremic dehydration and rhabdomyolysis after intense physical exercises [14]. The effectiveness of carbonic anhydrase may be also altered by autoantibodies. Ignaki et al. demonstrated in 1991 that anti-carbonic anhydrase autoantibodies (aCAAs) are present in serum of 32% patients with systemic lupus erythematosus, in 21% of patients with Sjögren's syndrome, but not in healthy volunteers. They showed that aCAAs have affinity to epidermal cells, hair follicles, sweat glands, and renal tubular cells [15]. Moreover, in 2007, it was presented that also in rheumatoid arthritis there are present aCAAs in serum, showing affinity to bind to carbonic anhydrase III in synovial membranes [16]. Up to date aCAAs have been described in patients with rheumatoid disorders (rheumatoid arthritis, Behçet's disease, lupus erythematosus, polymyositis, systemic sclerosis, and Sjögren syndrome) [16–18], digestive tract disorders (idiopathic chronic pancreatitis, primary biliary



10	20	30	40	50
MSHHWGYGKH	NGPEHWHKDF	PIAKGERQSP	VDIDTHTAKY	DPSLKPLSVS
60	70	80	90	100
YDQATSLRIL	NNGHAFNVEF	DDSQDKAVLK	GGPLDGTYRL	IQFHFHWGSL
110	120	130	140	150
DGQGSEHTVD	KKKYAAELHL	VHWNTKYGDF	GKAVQQPDGL	AVLGIFLKVG
160	170	180	190	200
SAKPGLQKVV	DVLDSIKTKG	KSADFTNFDP	RGLLPESLDY	WTYPGSLTTP
210	220	230	240	250
PLLECVTWIV	LKEPISVSSE	QVLKFRKLNF	NGEGEPEELM	VDNWRPAQPL
260				
KNRQIKASFK				

Figure 1: The structure and amino acid sequence of the human carbonic anhydrase II [29].

cirrhosis, autoimmune cholangitis, and gastric cancer) [18, 19], endometriosis [18], Grave's disease [20], acute myeloid leukaemia [21], renal tubular acidosis [22] (significant influence on pathogenesis proved in the animal model of Sjögren's syndrome [23]), and end-stage kidney disease [24]. Moreover, autoantibodies against carbonic anhydrase II were proved to play an important role in pathogenesis of retinopathy [25] and these against CAVI seem to induce dry eye syndrome in Sjögren's syndrome [26, 27]. Interestingly, the autoantibodies against carbonic anhydrase II might be produced in humans due to cross-reactivity with carbonic anhydrase of Helicobacter pylori [28].

Carbonic anhydrases (CAs) have become an interesting enzyme for clinicians when first drugs inhibiting this enzyme established promising group of diuretics. Acetazolamide was introduced into clinical practice in 1956 as a first nonmercurial diuretic. Among "classic" carbonic anhydrase inhibitors also methazolamide, ethoxzolamide, and dichlorphenamide were applied in congestive heart failure treatment. Nowadays, more preferable are the next-generation diuretics (loop, thiazide, thiazide-like diuretic, and aldosterone antagonists), but most of them still exhibit different magnitude of carbonic anhydrase inhibition [38]. Acetazolamide remains a useful drug in case of intracranial hypertension [39], improving sleep quality (reducing the apnea-hypopnea index in both obstructive sleep apnea and healthy trekkers) [40] and reducing the risk of mountain sickness at high altitudes [41]. Topical dorzolamide and brinzolamide and in some cases systemic acetazolamide are commonly used for reduction of intraocular pressure in glaucoma [42]. Topiramate and zonisamide are anticonvulsants with a multiple mechanism of action among which is also carbonic anhydrase inhibition. Not only does this effect seem to be important in the main (antiepileptic) activity of this drug, but also it might result in weight loss. It is regarded as a side effect but may be the basis for designing the new antiobesity drugs [38]. Moreover, acetazolamide is also proved to be effective in seizure treatment [43]. Induced by hypoxia, overexpression of carbonic anhydrase IX in neoplastic cells may produce acidosis and thus reduce the effectiveness of chemotherapies [38]. Inhibition of carbonic anhydrase IX improves effects of cisplatin in small cell lung cancer [44].

2. Carbonic Anhydrase II

Carbonic anhydrase II (CAII) is a cytoplasmic enzyme with a very high affinity for sulphonamides and high catalytic activity (for structure see Figure 1.). It is one of the fastest, working enzymes in the human body, 10^6 cycles of enzyme per second [45]. CAII is present in numerous tissues (e.g., erythrocytes, eye, gastrointestinal tract, bone osteoclasts, kidney, lung, testis, and brain) and it constitutes a possible drug target in some diseases (glaucoma, oedema, epilepsy, and attitude sickness). However, so far it has been mostly studied in the red blood cells and has been extracted from them for the purpose of *in vitro* studies [5].

The gene for human CAII is located on the chromosome 8q22 [46]. The diseases associated with CAII include the following: Autosomal Recessive Osteopetrosis Type 3 with Renal Tubular Acidosis (ARO3, OPTB3), which commonly

manifests in early infancy with macrocephaly, feeding difficulties, evolving blindness and deafness, bone marrow failure, severe anaemia, and hepatosplenomegaly. Deafness and blindness are generally thought to represent effects of pressure on nerves. OPTB3 is associated with renal tubular acidosis and cerebral calcification (marble brain disease) and in some cases with mental retardation [47]. Among its related pathways is vitamin D-receptor mediated regulation of genes involved in osteoporosis [48].

Numerous compounds have been demonstrated to modify carbonic anhydrase activity. They are physiological substances, drugs currently used in clinical practice and other chemicals tested *ex vivo* in laboratories. Potential CAII activators physiologically present in human organism include biogenic amines (histamine, catecholamines, and serotonin) and amino acids (phenylalanine and histidine) [49]. Most drugs inhibiting carbonic anhydrase are sulphonamides (over 20 FDA approved drugs including diuretics like hydrochlorothiazide, indapamide, chlortalidone, and furosemide) [50, 51]. There is a wide variety of substances testes in laboratory conditions for their CA activatory and inhibitory properties [5, 52].

The aim of this review article was to collect available data regarding studies on carbonic anhydrase in platelets and concerning the influence of carbonic anhydrase regulators on platelet function. Taking into account limited number of studies directly analyzing carbonic anhydrase properties in platelets, a secondary objective was to analyze the involvement of drugs regulating CAII in platelet pathophysiology and to discuss hypothetical contribution of platelet carbonic anhydrase in these drug-platelet interactions.

3. Platelet Carbonic Anhydrase

The first statements of CAII in platelets were made 60 years ago [53] and its more accurate characterization was created in the '80s of the last century [54]. CAII, by catalysing the formation of H^+ and HCO_3^-, reduces the cytosol pH of the platelet [55]. The reaction products can be excreted outside the plasma membrane – H^+ is being exchanged for Na^+ whereas HCO_3^- for Cl^- (Figure 2).

The exact role of CAII in platelet physiology requires more detailed research. A question arises whether their products play a direct role in platelet physiology or enhance the transmembrane ion exchange, sodium and chloride influx. So far there has been no clinical case presented in the PubMed database that describes CAII deficiency in platelets and, consequently, the effects of this deficiency on the phenotype. The only available case is the description of a murine mutation located in the direct proximity to the CAII gene locus, which affected both platelet morphology and function [56].

The CAII activity in platelets has been hypothesized since Akkerman et al. described proton efflux from platelets following thrombin stimulation [57, 58]. Siffert et al. verified the presence of CO_2 hydration in platelets, subsequently proved that this process may be inhibited by ethoxzolamide, and showed that examined enzyme kinetics strictly corresponds with carbonic anhydrase II [54]. Afterwards, ethoxzolamide

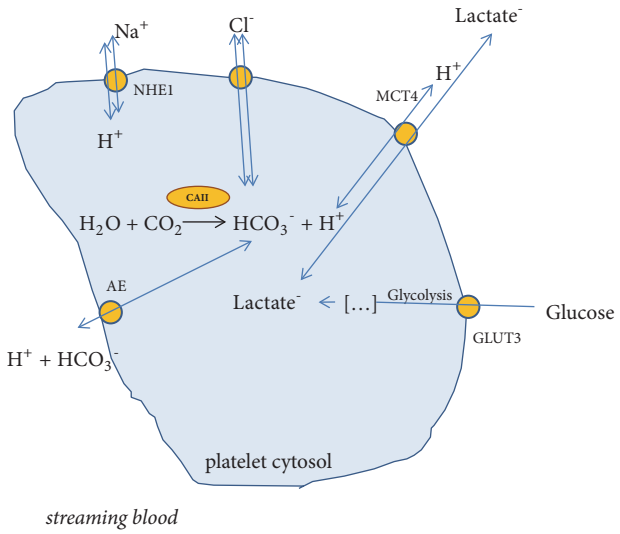


Figure 2: A potential role of carbonic anhydrase in the regulation of platelet metabolism [30–32]. *GLUT3: glucose transporter; MCT4: H+/monocarboxylate transporter; AE: Anion exchanger; NHE1: the Na+/H+ exchanger.*

was shown to reduce the thrombin stimulated aggregation by about 20%-40% [59, 60]. Similar result was achieved by the removal of CO_2 from platelets environment, which additionally confirms the importance of carbon dioxide hydration in the aggregation process [59].

3.1. Effect of Carbonic Anhydrase Activators on Platelet Function. Adrenaline is CAII agonist [61] and it can sensitize platelets to thrombin by induction of CAII activity [62, 63]. Adrenaline stimulates platelet carbonic anhydrase directly or by enhancing the membrane HCO_3^-/Cl^- exchange since such subthreshold doses of adrenaline (in concentrations which do not initiate aggregation) added to platelets are capable of increasing the chloride concentration in platelet cytosol. [55]. Not only does a CAII inhibitor, acetazolamide, reduce resting and adrenaline-induced chloride concentration in platelets, but it also abolishes the synergism of thrombin and low concentration of adrenaline [63]. The proaggregatory effect of adrenaline is also abolished by another CAII inhibitor, chlortalidone. Furthermore, the ability of adrenaline to initiate platelet aggregation has been demonstrated to be directly proportional to the activity of platelet carbonic anhydrase [64].

Serotonin is an activator of carbonic anhydrase [49], which is also a poor agonist for platelet aggregation [65]. Interestingly, it enhances adrenaline, adenosine diphosphate (ADP) and collagen induced aggregation [65]. Furthermore, while being hyperreactive to adrenaline alone or adrenaline+serotonin, platelets present increased binding affinity for serotonin [65]. This observation may be clinically important as in an *in vivo* dog model serotonin plasma level was increased in stress conditions and it was suspected to significantly interplay with the evolution of an occlusive coronary thrombus [66], which was verified in human observation trial where high plasma serotonin level was associated

with presence of coronary artery disease and future cardiac events [67]. This effect seems to be mediated through platelet 5-HT (5-hydroxytryptamine) receptors as saprogelate (a 5-HT_2 receptors antagonist) limits platelet aggregation [68–70]. To the best of our knowledge, the possible participation of direct serotonin influence on platelet carbonic anhydrase has not been evaluated.

The selective serotonin reuptake inhibitors (SSRI), fluoxetine, sertraline, and citalopram, are potent activators of carbonic anhydrase II [71] and therefore they may be suspected of proaggregatory properties. Nevertheless, another SSRI, paroxetine, decreases intraplatelet serotonin storage and at the same time lowers platelet activation [72]. Similarly, sertraline and citalopram also limit platelet aggregation [73, 74]. Fluoxetine was to decrease platelet aggregability in the case of a 49-year-old man [75]. Therefore, SSRI effect on platelet function may be the balance between their direct action on platelets and the result of decreased platelet serotonin content. Nevertheless, the impact of this group of drugs on both thrombotic [76] and bleeding events is not substantial [77].

Histamine is a poor carbonic anhydrase II activator [52] present in humans platelets and it plays an important role in modulating platelet function. Histamine does not initiate platelet aggregation by itself, but it increases platelet sensitivity to aggregation agonists like ADP, thrombin, collagen, arachidonic acid [78], and adrenaline [79]. In a clinical model of increased histamine release, patients with chronic urticaria, higher platelet activity was observed when compared to healthy controls: increased levels of soluble P-selectin in one study [80] and both enhanced aggregation in response to ADP and increased soluble P-selectin levels compared to healthy population in another paper (both parameters were strongly and positively correlated with the Urticaria Severity Score) [81]. The mechanism of histamine impact on platelet pathophysiology is not fully understood. Histamine proaggregatory properties seem to result from H_1 receptor stimulation (located in platelet membrane [82]). However, involvement of histamine in intracellular metabolism is also suggested [78], which was the subject matter of some studies [79, 83, 84]. Interestingly, there are no studies verifying whether CA inhibitors attenuate histamine affect platelet aggregation and if such an inhibition may be useful, e.g., in chronic urticaria management.

Histidine, phenylalanine, and carnosine (β-alanine-histidine dipeptide) are carbonic anhydrase activators [52]. Data regarding effect of histidine and carnosine on platelet function are not consistent. In some studies, histidine was verified to be an inhibitor of ADP-induced platelet aggregation [85] or spontaneous platelet aggregation and thromboxane B_2 (TxB_2) formation [86], but in others it demonstrated both slight proaggregative activity (50% of the patients) and antiaggregatory activity (the other 50% of patients) [87]. Similarly, in one study carnosine slightly stimulated ADP-induced aggregation [87], but in another it enhanced platelet aggregation only in patients with a low rate of aggregation, but inhibited platelet aggregation in patients with high index of aggregation [88]. There are hardly any available data on phenylalanine influence on platelet function; in a single study phenylalanine methyl ester inhibited ADP-induced platelet aggregation. Interestingly, similar results were observed with histidine methyl ester, but not with pure histidine (no inhibition) [89].

There are no available papers analyzing histidine, phenylalanine, and carnosine influence on CAII in platelets. Nevertheless, while analyzing their action profile, they do not appear to act on platelets through CAII.

3.2. Diuretics as CAII Inhibitors. Systemic use of acetazolamide is dedicated for altitude sickness treatment and prevention. Primarily, it reverses hypocapnic alkalosis occurring due to ventilatory response to hypoxemia, but the exact mechanism of its beneficial action seems to be more complex and is not fully recognized [41]. Moreover, in people exposed to high altitudes, thrombosis is a proposed mechanism of several complications as in autopsy many megakaryocytes were present in the lungs of people diagnosed with pulmonary edema and in one case they were also accompanied by thrombi in the kidneys and liver [90]. Nevertheless, at high altitude platelets present increased adhesiveness [91], but decreased aggregation in response to ADP, adrenaline, and collagen [92, 93] so eventual benefit of acetazolamide antiaggregatory properties remains uncertain.

Diuretics commonly used in clinical practice (hydrochlorothiazide, chlortalidone, indapamide, furosemide, and bumetanide) are well-established carbonic anhydrase inhibitors [51]. Thiazide-like diuretics, indapamide and chlortalidone, are widely used in cardiovascular medicine. They are proved to prevent cardiovascular events (CVE) and reduce all-cause mortality. The CVE prophylactic effect exceeds the benefits of lowering blood pressure. The difference was revealed when this thiazide-like diuretics were compared with thiazide-type diuretics [94]. The important differences in activity between these two groups of drugs regard platelet inhibition, which is much more intense in thiazide-like than thiazide-type diuretics as shown in work by Rendu at al. comparing indapamide and hydrochlorothiazide properties [95]. Furthermore, the platelet inhibition by thiazide-like diuretics seems to be carbonic anhydrase dependent [64].

Loop diuretics, furosemide and bumetanide, are loop diuretics potent to inhibit carbonic anhydrase II activity [51]. In one clinical study, furosemide was shown to inhibit ADP-induced platelet aggregation both ex vivo and after intravenous infusion [96] and in another study furosemide inhibited *in vitro* both ADP- and AA-induced aggregation [97]. Bumetanide was also shown to inhibit adrenaline-induced aggregation [63]. The mechanism of influence of loop diuretics on platelets remains unclear and the involvement of carbonic anhydrase may be expected.

3.3. Physiological Nitrogen Compounds versus Platelet Carbonic Anhydrase II Function. Platelet aggregation may be inhibited by nitric oxide (NO). However there is a controversy about possible significance of this dependence since the extra-platelet NO concentration is low and it seems to be no expression of nitric oxide synthase in platelets [33, 34]. Nevertheless, the effect of NO on platelets is mediated

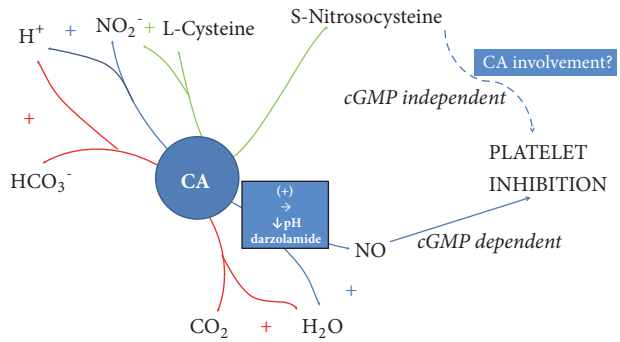


Figure 3: Postulated map of carbonic anhydrase II activities involving nitrosothiols, nitrate, and nitric oxide metabolism and they influence on platelet aggregation. S-Nitrosocysteine and NO are established platelet aggregation inhibitors [33, 34]. S-Nitrosocysteine is able to act independently of cGMP formation [35]. Dorzolamide and decreased pH increase nitric oxide formation [36]. CA ability to form NO from nitrite inside platelets remains controversial [36]. Nevertheless, $NaHCO_3^-$ + NO_2^- in presence of erythrocytic CA II provides platelet cGMP formation [36] (more detailed description in the main text).

through the activation of guanylyl cyclase to produce cyclic guanosine monophosphate (cGMP) [98, 99]. Interestingly, carbonic anhydrase II was found to catalyse generation of nitric oxide form nitrite [100], which may be an additional origination of intra-platelet NO. This reaction is significantly enhanced by both decreased pH and dorzolamide, which are inhibitors of CAII main activity [100]. On the other hand, other research groups provide evidence negating this CA activity [36].

Nitrite is a platelet inhibitor in whole blood [101], but added to platelets suspended in platelet rich plasma it does not inhibit platelet aggregation [102] or activate soluble guanylate cyclase (sCG) [36]. It does not produce increased intraplatelet nitrite concentration [103], either. Therefore it (in these conditions) cannot rich cytosolic enzymes like carbonic anhydrase II.

Platelet supplementation with S-Nitrosocysteine (SNC), a NO donor, also provides intraplatelet NO_2^- formation and leads to platelet sCG activation [35, 36, 103]. Interestingly, SNC aggregation inhibition is not mediated by sCG activation. SNC also presents direct cyclooxygenase (COX) inhibiting properties, but an involvement of one more mechanism is postulated as it produces complete blockage of AA-induced aggregation, when TxB_2 formation is only partially decreased [103]. Therefore, SNC is supposed to be involved in one more mechanism modulating platelet function, which may be CA. This thesis of CA contribution may be supported by observation that SNC produces increased intraplatelet nitrite formation [103]. CA II is proven to catalyse opposite reaction; it can produce S-nitrosothiols from inorganic nitrite [36] (Figure 3.).

Nitrate is able to bind to carbonic anhydrase active site [104], but not it cannot be utilized to form S-nitrosothiols as nitrite can be [105]. It can inhibit platelet aggregation in whole blood, but the mechanism remains unclear [101].

3.4. Other Drugs Potentially Inhibiting Platelet Carbonic Anhydrase II. An experiment considering influence of N-hydroxyurea on platelet activity was performed by Lahiri at al. in chronic myelogenous leukaemia. There were at least three patterns of response to hydroxyurea, no response, partial aggregation inhibition, and "de-aggregation". Interestingly, both aggregation inhibition and "de-aggregation" were only partially reversible by ODQ (1H-(1,2,4)Oxadiazolo(4,3-a)quinoxalin-1-one), which indicates involvement of other mechanism than postulated by authors NO release stimulating cGMP formation [106]. The inhibition of platelet carbonic anhydrase II by N-hydroxyurea may be possible [107].

Coumarin and its derivates are carbonic anhydrase inhibitors and substrates [108, 109]. Unfortunately, there is no information on acenocoumarol and warfarin (two coumarin derivates widely used as anticoagulant drugs [110]) regarding their individual influence on carbonic anhydrase. Nevertheless, these two drugs do not seem to inhibit platelet carbonic anhydrase in humans as both of them enhance platelet activity [111, 112].

A substantial part of nonsteroid anti-inflammatory drugs (NSAIDs) present anti-CAII activity. Celecoxib [113, 114] and valdecoxib [115], but not rofecoxib [114], meloxicam, piroxicam, and lornoxicam [116], are carbonic anhydrase II inhibitors. Diclofenac, which does not possess sulphonamide moiety, is not active against CA [116]. Flurbiprofen is a weak carbonic anhydrase II inhibitor whereas ibuprofen and indomethacin are even much less active against this enzyme [117]. In two papers by Puscas et al. indomethacin was said to be an agonist of CA, but this conclusion was reached after indomethacin limited inhibitory effect of acetazolamide on carbonic anhydrase [118, 119] and it was uncertain whether it was indeed CA agonist or just weaker than acetazolamide CA inhibitor competing for a binding site. Acetylsalicylic acid (ASA) is a noncompetitive carbonic anhydrase II inhibitor [120], but this activity may be associated with adjusting environmental pH by ASA [121]. The effect of other NSAID on platelet function does not seem to depend on CAII regulation, but exclusively on COX-1 inhibition. A nonspecific COX inhibitor naproxen, but not COX-2 selective inhibitor celecoxib inhibits both collagen and arachidonate induced platelet aggregation [122]. Nevertheless, in a recent report we provided evidence that interindividual variability in *in vitro* platelet responsiveness to acetylsalicylic acid may be associated with carbonic anhydrase II concentration. Briefly, ASA low-responders presented increased intraplatelet CAII concentration and more intense baseline arachidonic acid induces aggregation compared to ASA sensitive individuals [123]. Among the CAII-dependent mechanisms modifying the platelet responsiveness, the pH changes of platelet cytosol leading to impaired acetylating of cyclooxygenase by ASA are noteworthy. This in turn could affect the antiplatelet effect of ASA as well as platelet inflammatory activity and energetic metabolism (Figure 4).

To conclude, the role of carbonic anhydrase II in cardiovascular medicine is still underestimated and requires further in-depth studies.

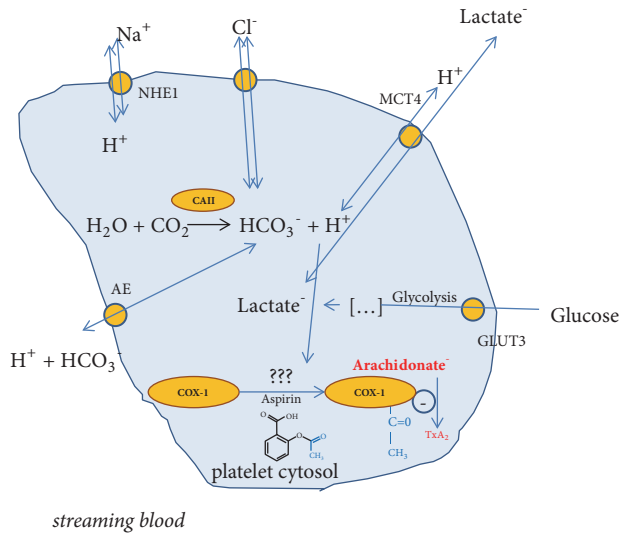


FIGURE 4: A potential role of carbonic anhydrase II (CAII) in regulating ASA response [30–32, 37]. *GLUT3: glucose transporter; MCT4: H+/monocarboxylate transporter; AE: anion exchanger; NHE1: the Na+/H+ exchanger; COX-1: cyclooxygenase-1; TxA_2: thromboxane A_2.*

4. Conclusions

Even though there is a well-documented rationale for an important role of carbonic anhydrase II in regulating platelet function, its exact role in platelet physiology and pathology remains poorly understood. A more frequent use of platelet CAII inhibitors holds promise as a good strategy to reduce the impact of cardiovascular diseases. However, future prospective clinical studies, supported by the evidence-based medicine principles, are needed in order to precisely elucidate the role of platelet CAII in cardiovascular medicine.

There is a need for more basic scientific investigations in order to establish the role of platelet carbonic anhydrase II in the pathogenesis of several diseases such as chronic urticaria and altitude sickness and, further, to verify the contribution of platelet CAII in metabolism of nitrites and antiaggregatory properties of S-Nitrosocysteine.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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