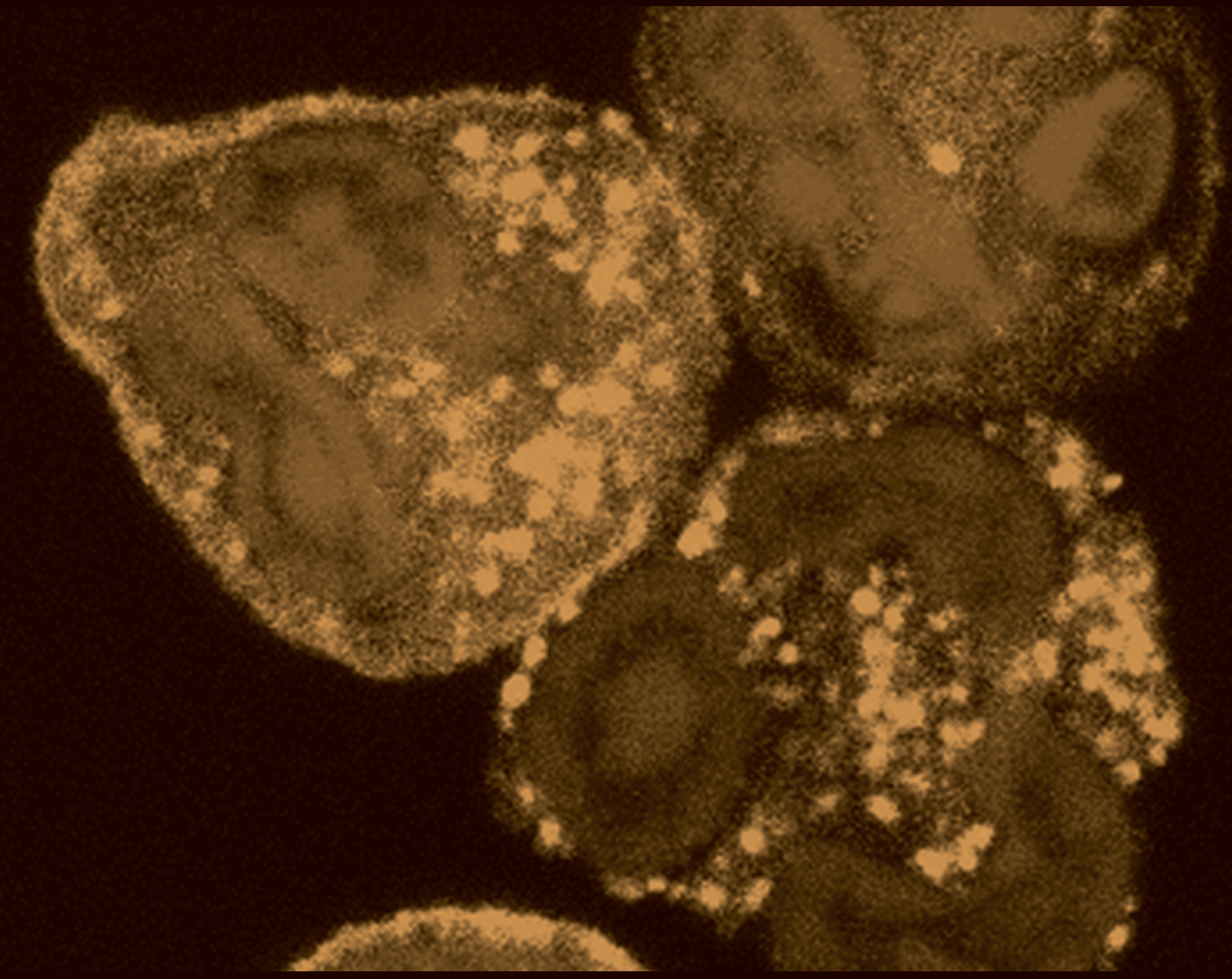
Mediators of Inflammation

Inflammatory Cardiovascular Risk Biomarkers: Update on Novelties and Limitations

Guest Editors: Fabrizio Montecucco, François Mach, Aldo Pende, Thomas H. Schindler, Rafaela F. da Silva, and Nicolas Vuilleumier



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Editorial Board

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Editorial

Inflammatory Cardiovascular Risk Biomarkers: Update on Novelties and Limitations

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Evidence from Framingham studies showed that some disorders and conditions (such as hypertension, hyperlipidaemia, smoking, diabetes, old age, and male sex) are particularly useful to estimate the cardiovascular (CV) risk of acute ischemic events [1] and are currently considered as the "traditional" cardiovascular risk factors. This led to the development of several clinically based CV risk stratification tools, and the Framingham risk score is one of the most commonly used CV risk stratification tools nowadays [2]. However, these "traditional" cardiovascular risk factors were shown to be suboptimal for proper CV risk stratification due to low specificity and sensitivity [3–6]. Therefore, a novel concept of "global" cardiovascular vulnerability has been suggested to better predict acute cardiovascular events [7, 8]. Interestingly, this approach was particularly focused on "the clinical and laboratory complexity of the patient" instead of "a single risk factor." Several pathophysiological parameters have been proposed as new cardiovascular risk factors potentially improving the assessment of patient vulnerability [7, 8]. Although some controversies still exist, the atherosclerotic role of inflammatory biomarkers (such as C-reactive protein (CRP), cytokines, and chemokines) [9–12] has been shown within atherosclerotic plaques, in the systemic circulation or in the peripheral ischemic tissues in both *in vivo* and *in vitro* models. More recently, novel inflammatory mediators (such as circulating autoantibodies and hormones) have been also identified [13, 14]. These soluble mediators have been shown to trigger several atherosclerotic functions of both inflammatory and vascular cells [15]. On the other hand, the mobilization of protective cell subsets might also counterbalance atheroprogression, thus limiting the chronic inflammatory processes and improving cardiovascular outcomes [16]. These protective aspects might be particularly relevant when atherosclerosis is associated with concomitant inflammatory conditions (such as rheumatoid arthritis, infections, and diabetes), which seem to further accelerate atherogenesis towards final acute ischemic complications or arterial aneurysms [17–19]. This special issue focused on new soluble mediators as promising candidates to better assess the cardiovascular risk. Importantly, the limitations of the potential clinical use of these systemic and intraplaque inflammatory molecules influencing atheroprogression have also been discussed. In particular, E. Lupia and coworkers revised the clinical role of thrombopoietin (a humoral growth factor activating platelets) as a promising biomarker of cardiovascular injury. P. Kunes and colleagues developed interesting findings on the controversial role of pentraxin family (which includes CRP) in the inflammatory response. The authors focused on

the newly discovered pentraxin 3 and suggested paradoxical issues that will probably be validated in the near future. Drs. D. Vasic and the D. Walcher from the University of Ulm (Germany) revised the potential proatherosclerotic activity of C-peptide as a predictor of cardiovascular risk in diabetic subjects. This paper focused on a hot-topic issue in cardiovascular research. In fact, only very recently, C-peptide (previously considered as a product of cleavage of proinsulin) has been proposed as an active factor favouring atherosclerosis. Despite some limitations on the molecular mechanisms (the C-peptide receptor remains to be identified), this molecule might activate different leukocyte subsets in atherogenesis. Among these cells, the different types of macrophages appear as a relevant target for inflammatory mediators. T. Gui and coworkers discussed this issue and provided an interesting and comprehensive review on the impact of macrophages in both early and advanced phases of atherogenesis. The authors also suggested these cells as promising biomarkers of plaque vulnerability. Another review article of the present issue mainly focused on the proteolytic mechanisms regulating intraplaque remodelling potentially favouring the formation of abdominal aortic aneurysms. Drs. Z.- Z. Li and Q.-Y. Dai focused on direct activities mediated by nicotine via its receptor (nicotinic acetylcholine receptor) on plaque inflammation, angiogenesis, and smooth muscle cell dysfunction. The paper of Z. Qu and colleagues further developed the proinflammatory reactions underlying abdominal aortic aneurysm formation in advanced atherosclerosis. Some receptors of sphingosine-1-phosphate (S1P, a recently discovered lysophospholipid) were shown to play a crucial role in human abdominal aorta aneurysms as compared with normal aorta control tissues. In particular, S1P3 receptor was significantly upregulated in human abdominal aortic aneurysms, while S1P2 receptor was downregulated as compared to normal aortic samples. Although the molecular mechanisms remain unexplored (parallel expression of other inflammatory mediators was not investigated), this observational study identified a potential novel cardiovascular biomarker (S1P) in advanced atherosclerosis. Differently from this paper, K.-Karatolios and coworkers focused their study on well-known cytokines and growth factors. Surprisingly, the authors showed that the levels of vascular endothelial growth factor (VEGF) and human basic fibroblast growth factor (bFGF) in pericardial effusions of patients with autoreactive or viral inflammation were significantly higher as compared to patients with coronary artery disease (CAD). No significant difference was shown for inflammatory cytokines. Although the underlying molecular mechanisms remain to be investigated, these two growth factors might be more promising biomarkers of pericardial inflammation than "traditional" cytokines. After a diffuse discussion on soluble mediators potentially increasing the cardiovascular risk, R. Wyderka and coworkers focused their investigation on the mobilization of protective CD34+CXC4+ stem/progenitor cells in humans after an acute myocardial infarction. The authors clearly showed that this process was positively correlated with the improvements of values of the left ventricular ejection fraction at 1-year of followup, suggesting a beneficial activity of these cells in myocardial repair.

In both inflammatory micro- and macroenvironments characterizing atherosclerosis, some tissues might also produce some unexpected molecules potentially contrasting with physiological paradigms. M. L. Sirico and coworkers showed that human adipocytes can express and synthesize albumin. This paper was selected to highlight the potentialities of adipose tissue as an inflammatory organ capable of ectopically producing a large variety of mediators during atherogenesis. The present issue includes by the paper of C. Falcone and colleagues investigating the potential activity as biomarkers of soluble Receptor for Advanced Glycation End products (sRAGE) in patients with hypertension and increased cardiovascular risk. The authors showed that antihypertensive treatments might affect sRAGE plasma levels. All the papers included in the present issue focused on both novelty and limitations of promising inflammatory biomarkers that in the near future might be used in the clinical practice to improve cardiovascular disease prevention. We hope that the reader will find some useful inputs for developing research and updating knowledge on cardiovascular pathophysiology.

Acknowledgments

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Clinical Study

Human Mature Adipocytes Express Albumin and This Expression Is Not Regulated by Inflammation

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Aims. Our group investigated albumin gene expression in human adipocytes, its regulation by inflammation and the possible contribution of adipose tissue to albumin circulating levels. *Methods*. Both inflamed and healthy subjects provided adipose tissue samples. RT-PCR, Real-Time PCR, and Western Blot analysis on homogenates of adipocytes and pre-adipocytes were performed. In sixty-three healthy subjects and fifty-four micro-inflamed end stage renal disease (ESRD) patients circulating levels of albumin were measured by nephelometry; all subjects were also evaluated for body composition, calculated from bioelectrical measurements and an thropometric data. *Results*. A clear gene expression of albumin was showed in pre-adipocytes and, for the first time, in mature adipocytes. Albumin gene expression resulted significantly higher in pre-adipocytes than in adipocytes. No significant difference in albumin gene expression was showed between healthy controls and inflamed patients. A significant negative correlation was observed between albumin levels and fat mass in both healthy subjects and inflamed ESRD patients. *Conclusions*. In the present study we found first time evidence that human adipocytes express albumin. Our results also showed that systemic inflammation does not modulate albumin gene expression. The negative correlation between albumin and fat mass seems to exclude a significant contributing role of adipocyte in plasma albumin.

1. Introduction

Adipose tissue, once just believed a passive organ storing excess energy, is now considered a real endocrine organ, with an important role in the regulation of homeostatic systems [1]. It secretes various proteins that can be classified into molecules acting on metabolic processes like glucose homeostasis and insulin sensitivity, and into inflammatory molecules like interleukin 6 (IL-6), interleukin 1β, Tumor Necrosis Factor-α, C-reactive protein (C-RP), Leptin, and Adiponectin [2]. The adipose tissue may account for 20–25% of systemic IL-6 circulating levels [3] and seems to be involved in the systemic inflammatory response associated with obesity, insulin resistance, and metabolic syndrome [4].

On this basis, adipose tissue represents a new and fascinating multidisciplinary object of research. We previously found a clear expression of IL-6 receptors and C-reactive protein in human adipocytes obtained from adipose tissue fragments of different districts (subcutaneous and omental of noninflamed and inflamed patients), showing that the adipose tissue, and in particular the adipocyte, was not only responsible for generating an inflammatory state per se but it also represents a target, by itself, of systemic inflammation [5]. We observed, in fact, that the adipocyte, stimulated by IL-6 through its cell receptor produces C-RP, as it happens in the hepatocyte.

Adipose tissue is a mesodermallyderived organ that contains, in addition to adipocytes, a stromal population

composed by different cell types, such as pre-adipocytes, stem cells, fibroblasts, and macrophages. Mature adipocytes account for only 40–60% of the whole cell population whatever the type of adipose tissue [6]. Pre-adipocytes are present throughout adult life in adipose tissue and can proliferate and differentiate into mature adipocytes, according to the energy balance; they also share some features with macrophages, such as the capacity of phagocytosis in response to different stimuli [7]. Another important cell population of the adipose tissue is represented by mesenchymal stem cells (MSCs) that are adherent, fibroblast-like, pluripotent, and nonhematopoietic progenitor cells. These cells retain the capacity to undergo into many mesenchymal cell types, including bone cells [8, 9], neuronal cells [10], adipose [11], and muscular [12] tissue *in vitro*, and of note, into hepatocyte-like cells [13–15]. Thereby, both the pre-adipocyte, with a very restricted potential, and the multipotent mesenchymal cell represent adipocyte precursors.

Albumin, normally produced by the hepatocyte, is the most abundantly circulating protein in blood, where it plays an important role as binding protein and is involved in the regulation of colloid-osmotic homeostasis [16]. Circulating half-life of plasma albumin is 19 to 21 days. It is also a negative acute phase protein and hypoalbuminemia is the result of the combined effects of inflammation, through inhibitory cytokine (especially IL-6), and inadequate protein and caloric intake in patients with chronic inflammatory disease, such as chronic renal failure [17] where it represents a cardiovascular risk factor [18].

Considered the well-known active role of adipocyte in C-RP production and the presumable link between adipocyte and hepatocyte, we aimed this study at investigating if mature adipocytes express the gene of albumin and at evaluating whether systemic inflammation might regulate this expression, as we previously found for C-RP [5]. Furthermore, we speculated whether the adipocyte might contribute to the total circulating levels of albumin, as it happens for IL-6.

2. Methods

2.1. Quantization of Albumin mRNA and Protein Presence in Adipocytes and Preadipocytes

2.1.1. Patients and Healthy Controls Selection and Adipose Tissue Samples. Human subcutaneous adipose tissue samples were obtained from twelve subjects divided in two subgroups on the basis of a preliminary evaluation of C-RP circulating levels. According to Pepys and Hirschfield [19] we assumed a cut-off level of 3 mg/L, in order to discriminate between inflamed and noninflamed patients. On the basis of C-RP values, we obtained a group of six healthy (noninflamed) subjects, without any clinical symptoms or sign of inflammation, who underwent minor surgery procedures and a group of six patients with chronic inflammatory disease who underwent elective surgical procedures: (a) four for cancer and (b) two patients operated for cholecystectomy.

All enrolled subjects provided written, informed consent, and the Ethics Committee of our hospital approved the study.

To avoid a possible confounding inflammatory effect exerted by obesity or overweight status, inflamed patients and noninflamed controls were BMI matched in both groups of subjects. The presence of other systemic diseases (vasculitis, rheumatoid arthritis, osteoarthritis, and bowel or lung inflammatory disease) was excluded in all subjects (excepted malignancy); in particular, before the definitive inclusion, the possible existence of any immunological disease, malignancy (in the healthy group), and infectious disease was carefully investigated and excluded. No patient was diabetic and none assumed steroid or immunosuppressive therapy.

Blood samples were collected, before surgical procedures, from all studied subjects to obtain serum aliquots. Sera were stored at −80°C until C-RP and IL-6 assays were performed. Adipose tissue biopsies were obtained during the surgical procedures in all 12 subjects and for each patient was obtained a sample of subcutaneous total white adipose tissue and a sample of omental adipose tissue. After removal, to get rid of tissue debris the bioptic material was washed twice with warm sterile 0.9% NaCl solution; then the sample was immediately frozen in liquid nitrogen and stored at −80°C until RNA extraction.

2.1.2. C-Reactive Protein (C-RP) Assay. C-RP circulating levels were determined by a high-sensitivity ELISA assay (Bender MedSystems, Vienna, Austria) on serum aliquots of all subjects included in the study. The lower detection limit was 3 pg/mL, and the overall intra-assay coefficient of variation has been calculated to be 6.9%. All samples were analyzed in duplicate.

2.1.3. IL-6 Assay. The concentrations of IL-6 in plasma samples were analyzed by ELISA using a commercially available kit (Quantikine; R&D Systems, Minneapolis, MN), as described elsewhere [20]. The lower detection limit of IL-6 assay was <0.70 pg/mL, and the coefficient of variation of both inter- and intra-assay was <5%. All samples were analyzed in duplicate.

2.1.4. Isolation of Adipocytes, Stromal Cells, and Preadipocytes from Adipose Tissue Fragments. Adipose tissue fragments obtained from the six noninflamed subjects and from the six inflamed patients were placed in 37°C, sterile 0.9% NaCl, 5.6 mM glucose, and 25 mM HEPES buffer with pH adjusted to 7.4 and containing 50 U penicillin/mL plus 50 mg streptomycin/mL. The adipose tissue fragments were minced under sterile conditions and digested in Krebs-Ringer bicarbonate buffer supplemented with 5.6 mM glucose, 50 U penicillin/mL, 50 mg streptomycin/mL, and 17 mg type I collagenase/10 g adipose tissue (Worthington Biochemical, Lakewood, NJ). Digestion was for 75 min at 37°C, with rotary agitation at 40 rpm. The isolated cells were filtered through a single layer of chiffon, and the isolated adipocytes were allowed to float for 5 min at 37°C. The fluid under the floating adipocytes (containing stromal fraction) was transferred to a conical polypropylene 50-mL

centrifuge tube. For the adipocytes, 1 mL of cell suspension of adipose cells was suspended in each tube containing specific adipocyte medium (Zen-Bio, Research Triangle Park, NC) and incubated in the specific conditions until the cell lysis process. The stromal fraction was centrifuged at 800 g for 10 min.

For the isolation of pre-adipocytes, we first centrifuged the cell pellet at 800 × g for 10 minutes and then we washed three times with Dulbecco's modified Eagle medium : nutrient mixture F-12 (Ham) in combination 01 : 01 (DMEM: F12, Gibco-BRL, Carlsbad, California), which has been supplemented to give 15-mM NaHCO3, penicillin 50-U: mL streptomycin and 50 mg: mL. After washing, the pellet was resuspended by trituration in DMEM supplemented pre-adipocyte: F12 containing 10% fetal bovine serum (FBS, Gibco-BRL, Carlsbad, CA). The cells were then plated and incubated in a humidified incubator at 37°C in an atmosphere of 5% CO2 in air for 24 hours to allow attachment and proliferation of cells. After 24 hours, the medium was removed and replaced by specific pre-adipocyte medium (Zen-Bio, Inc., Research Triangle Park, NC) and subculture.

2.1.5. Identification of Primers for Albumin and β-Actin. The genome sequences corresponding to Albumin and β-Actin were obtained from GeneBank (http://www.ncbi.nlm.nih.gov/) to identify specific primers. We analyzed the exon sequences to establish a pair of primers (sense and antisense) able to generate amplified fragments measuring between 150 and 700 bp in length, with annealing temperature between 55 and 62°C. The sense and antisense primers were selected to include at least one intron to prevent genomic DNA contamination during amplification. The selected primer is shown in Table 1.

2.1.6. RNA Isolation and Expression Analysis by Reverse Transcription-Polymerase Chain Reaction (RT-PCR). The isolated cells were pulverized with a blender. Total RNA was extracted by the guanidinium thiocyanate technique. Four μg of total RNA were subjected to cDNA synthesis for 1 h at 37°C using the "Ready to go You-Primer First-Strand Beads" kit (Amersham Pharmacia Biotech, Piscataway, NJ, code 27-9264-01) in a reaction mixture containing 0.5 μg oligo-dT (Amersham Pharmacia Biotech cod. 27-7610-01). PCR amplification of cDNA was performed in a reaction mixture containing 4 μL of cDNA sample and different primer sets (20 p/mol each). The amplification of Albumin gene and human β-actin gene, as an internal control, was achieved using 2 primer sets. After an initial denaturation at 94°C for 5 min, PCR reactions were carried out using 30 cycles of 94°C for 1 min, the temperature for annealing for 1 min, and 72°C for 1 min in a Perkin Elmer Cetus 480 thermal cycler (Perkin Elmer). PCR products were separated by gel electrophoresis on a 2% agarose gel and stained with ethidium bromide. The bands obtained were quantified by densitometric analysis. All signals were normalized to the mRNA levels of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase and expressed as a ratio.

2.1.7. Real-Time PCR. Real-time quantitative PCR analysis for albumin gene was performed on cell lysate (both adipocytes and stromal fraction) using ABI prism 7500 (Applied Biosystems, Foster City, CA) and the 5-exonuclease assay (TaqMan technology). The cDNA, synthesized as described above, was used for real-time PCR performed in 96-well optical reaction plates with cDNA equivalent of 100 ng of RNA in a volume of 25 μL reaction containing 1x Taqman Universal Master Mix, optimized concentrations of FAM-labeled probe, and specific forward and reverse primer for Albumin gene from Assay on Demand (Applied Biosystems). Controls included RNA subjected to RT-PCR without reverse transcriptase and PCR with water replacing cDNA. The results were analyzed using a comparative method, and the values were normalized to the β-actin expression and converted into fold change based on a doubling of PCR product in each PCR cycle according to the manufacturer's guidelines, as previously described.

2.1.8. Western Blot. Homogenates of adipocytes and preadipocytes were prepared in radioimmune precipitation assay buffer (1X PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS containing phenylmethylsulfonyl fluoride, aprotinin, sodium orthovanadate and protease inhibitor tablet) (completeTM Mini, Boehringer-Mannheim) containing antipain dihydrochloride (50 mg/mL), bestatin (40 mg/mL), chymostatin (60 mg/mL), E-64 (10 mg/mL), leupeptin (0.5 mg/mL), pepstatin (0.7 mg/mL), phosphoramidon (300 mg/mL), Pefabloc SC (1 mg/mL), EDTA disodium salt (0.5 mg/mL), and aprotinin (2 mg/mL)]. The cells were centrifuged at 14,000 × g for 30 min at 4°C and protein concentrations were determined with the Bradford assay [21] using bovine serum albumin as a standard. For immunoblotting, protein (100 μg) was subjected to 8% SDS-polyacrylamide gel electrophoresis. Proteins were transferred to polyvinylidene difluoride (PVDF) membranes at a constant voltage of 200 V for 16 h. The PVDF membranes were blocked for 1 h in 5% nonfat dried milk in TBS-0.1% tween buffer (25 mM Tris-HCl, 0.2 mM NaCl; 0.1% Tween 20 (v/v) pH 7.6) (TBST). The membrane was washed 2X with TBST and then incubated overnight at 4°C with the respective primary antibodies. Albumin antibody was from Cell Signaling (Beverly, MA); GADPH antibodies were obtained from Santa Cruz Biotechnology. After extensive washing of membrane with TBST buffer, anti-rabbit immunoglobulin conjugated with horseradish peroxidase was added at a 1 : 5000 dilution and incubated for 1 h at room temperature. An enhanced chemiluminescence kit (Amersham, NJ) was used to identify protein expression.

2.2. Measurement of Albumin and Body Fat Mass in Healthy and Inflamed Patients. In sixty-three healthy (noninflamed) subjects and fifty-four microinflamed (C-RP: 5.24 ± 2.26 mg/L) ESRD patients undergoing RDT we determined circulating albumin levels (by nephelometry), Body Weight (BW) to the nearest 50 g (by using a calibrated balance beam scale), Body Mass Index (BMI), calculated as the

Table 1

Primers	Sense	Antisense
ALBUMIN	CTTGAATGTGCTGATGACAGG	GCAAGTCAGCAGGCATCTCAT
β-ACTIN	CACCATGGATGATGATATCG	TGGATAGCAACGTACATGG

Oligonucleotide sequences designed for this study.

ratio body weight/height^2 (in kg/m^2), and body composition, assessed by conventional bioelectrical impedance analysis (BIA) and by bioelectrical impedance vector analysis (BIVA), as previously described [22]. Resistance (R) and reactance (X_c) were measured by a single-frequency 50 kHz bioelectrical impedance analyzer (BIA 101 RJL, Akern Bioresearch, Firenze, Italy) according to the standard tetrapolar technique, by applying the software provided by the manufacturer, which incorporated validated predictive equations for total body water (TBW), fat mass (FM), fat free mass (FFM), and extracellular water (ECW) [23, 24]. The same investigators performed anthropometry and BIA measurements. Soft tissue hydration of individual subjects was evaluated by BIVA. R and X_c were normalized by the height of subjects (R/H and X_c/H) and the resulting vectors were plotted on a graph reporting the gender-specific 50th, 75th, and 95th tolerance ellipses of similar vectors calculated from a reference healthy population. According to the RX_c graph method, vectors falling within the reference gender-specific 75th tolerance ellipse indicated normal hydration; short vectors (below the lower pole of the 75th tolerance ellipse) indicated overhydration and long vectors (above the upper pole of the 75th tolerance ellipse) indicated underhydration [25]. The length of the vector was calculated as $|Z| = \sqrt{[(R/H)2 + (X_c/H)2]}$ and the phase angle of the vector as the arctan of X_c/R.

2.3. Statistical Analysis. Statistical analysis was performed by using unpaired t-test and ANOVA (followed by Bonferroni post hoc test) and linear regression analysis. Results are expressed as means ± SD; statistical significance was defined as $P < 0.05$.

3. Results

3.1. Quantization of Albumin mRNA and Protein in Adipocytes and Preadipocytes

3.1.1. Patients Selection and Adipose Tissue Samples. Demographic, anthropometric, and biochemical baseline data of all enrolled subjects are reported in Table 2. No significant difference was observed in sex, age, body weight, BMI, waist circumference, and waist-to-hip ratio between the two different groups. On the contrary, plasma C-RP and IL-6 levels were much higher in patients with chronic inflammatory diseases than in healthy (noninflamed) subjects ($P < 0.01$).

3.1.2. Albumin Gene Expression by RT-PCR in the Adipocyte and Preadipocyte. RT-PCR showed that the adipocytes extracted from all the districts of adipose tissue (omental and subcutaneous) of noninflamed controls and inflamed patients expressed the gene of albumin (Figure 1); the mRNA for this marker was found in the adipocytes and pre-adipocytes of all fragments of adipose tissue.

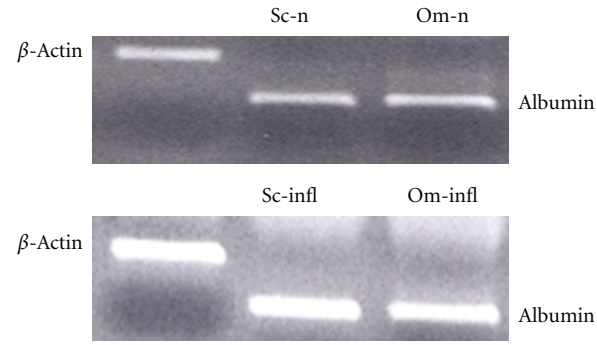

Figure 1: Albumin gene expression obtained by RT-PCR in subcutaneous (Sc) and Omental (Om) adipocytes from normal healthy subjects (Sc-n and Om-n, resp.) and inflamed patients (Sc-infl and Om-infl, resp.). β-Actin gene expression is also reported as housekeeping. The image is representative of all the experiments.

3.1.3. Albumin Gene Expression by Real-Time PCR in the Adipocyte and Preadipocyte. We did not find any statistically significant difference in albumin gene expression between inflamed and noninflamed patients in adipocytes drawn from both Sc and Om adipose tissue. As Figure 2 shows, we only observed an higher omental adipocyte albumin than subcutaneous one in each group. We concluded that inflammation does not modulate albumin gene expression in the adipocyte, but probably further studies are required to confirm this result. Figure 3 shows the different albumin gene expression, studied by real-time PCR, in adipocytes and pre-adipocytes obtained from either Sc or Om fragments of adipose tissue drawn from noninflamed subjects. Pre-adipocyte albumin gene expression was higher then the adipocyte one in both Om and Sc fragments and this difference was statistically significant. We also observed that albumin gene expression in the pre-adipocyte extracted from fragments of Om adipose tissue was significantly higher than in preadipocyte from Sc fragments. No difference was found between the adipocytes from different districts (Figure 3).

3.1.4. Albumin Protein Expression in the Adipocytes and Preadipocytes Obtained from the Different Types of Adipose Tissue Studied by Western Blot. Finally, we investigated whether at the protein level adipocytes and pre-adipocyte were also able to express albumin in all the subjects. As shown in Figures 4 and 5, Western Blot analyses showed the

Table 2: Demographic and anthropometric characteristics of the 12 subjects who underwent the adipose tissue biopsy.

	Noninflamed subjects	Chronic inflamed patients	*P* value
N	6	6	NS
Sex (M/F)	4/2	3/3	NS
Age, yr (range)	43.2 ± 4.0 (39–48)	42.9 ± 3.9 (34–49)	NS
Body weight, kg (range)	74.8 ± 10.6 (62.9–88.2)	75.3 ± 11.1 (63.2–90.7)	NS
BMI, kg/m^2 (range)	25.8 ± 1.6 (24.8–28.1)	26.3 ± 1.2 (24.6–28.7)	NS
Waist circumf., cm (range)	90.9 ± 4.6 (83–106)	92.1 ± 6.1 (79–108)	NS
Waist-to-hip ratio (range)	0.82 ± 0.11 (0.53–1.21)	0.88 ± 0.15 (0.65 ± 1.28)	NS
C-RP, mg/L (range)	2.18 ± 0.54 (1.6–3.0)	7.26 ± 3.26 (4.1–14.6)	$P < 0.01$
IL-6, pg/mL (range)	2.79 ± 1.26 (1.3–4.9)	25.19 ± 11.85 (10.3–40.3)	$P < 0.01$

Values are expressed as means ± SD; M, male; F, female; NS, not significant; C-RP, C-reactive protein.

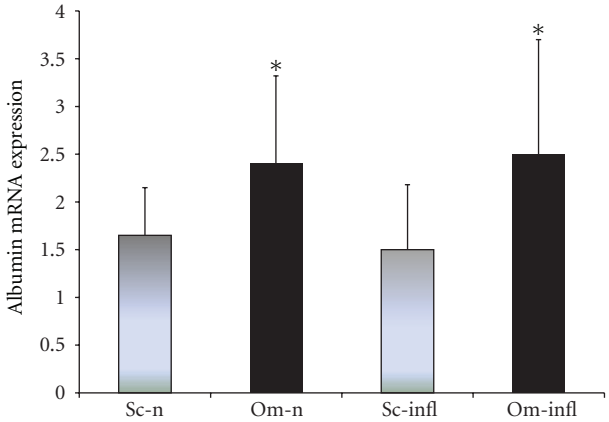

Figure 2: Albumin gene expression in the adipocytes from different sites of adipose tissue, obtained by real-time PCR analysis. The columns show the relative expression of albumin mRNA in subcutaneous (Sc, gray bars) and omental (Om, black bars) adipose tissue fragments of healthy noninflamed subjects (Sc-n and Om-n, resp.) compared with those obtained in both Sc and Om adipose tissue of inflamed patients (Sc-infl and Om-infl, resp.). The values on the *y*-axis represent arbitrary units derived from the mean expression value for albumin gene. Values are expressed in arbitrary units as means ± S.D. of 12 experiments. $^*P < 0.05$ compared with Sc-infl and Sc-n.

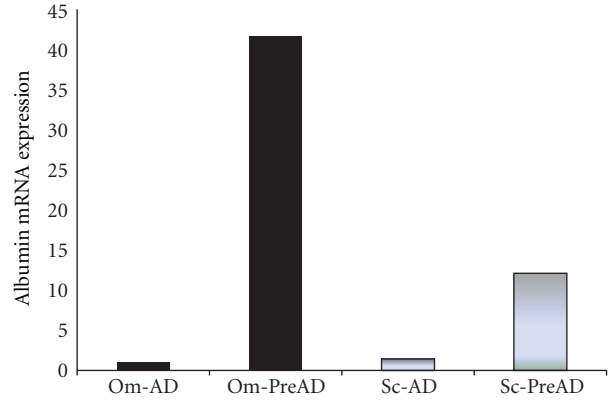

Figure 3: Albumin gene expression in the adipocytes and pre-adipocytes from different sites of adipose tissue of a single noninflamed subject, as representative of six experiments for all the subjects enrolled, obtained by real-time PCR analysis. The columns show the relative expression of albumin mRNA in Sc (gray bars) and Om (black bars) adipocyte (Sc-AD and Om-AD) and preadipocyte (PreAD) of a single healthy subject, as representative. The values on the *y*-axis represent arbitrary units derived from the mean expression value for albumin gene.

presence of albumin in both adipocytes and pre-adipocytes suggesting that both adipocytes and pre-adipocytes can synthesize albumin in Sc as well as in visceral adipose tissue, both in healthy noninflamed subjects and inflamed patients.

3.2. Correlations between Albumin Circulating Levels and Fat Mass in Both Noninflamed Subjects and Inflamed Patients. We first investigated the relationship between plasma Albumin levels and body fat mass (FM) in 63 healthy subjects who underwent BIA excluding from statistical analysis over- and hypohydrated subjects (vectors falling below the lower pole or vectors falling above the upper pole of the 75% tolerance ellipse) evaluated by BIVA method. As shown in Figure 6, a significant negative correlation was observed between plasma albumin levels and FM ($R = -0.312$, $P < 0.05$ (2-tailed)). Plasma albumin concentrations were significantly lower in those subjects with higher fat mass. Then, we studied the same relationship in 54 microinflamed ESRD patients undergoing regular dialysis therapy (RDT). As shown in Figure 7, a significant negative correlation was observed between plasma albumin levels and FM ($R = -0.391$, $P < 0.01$ (2-tailed)). Plasma albumin concentrations were significantly lower in those ESRD patients with higher fat mass.

4. Discussion

Albumin is the most abundant plasma protein produced by liver cells. To date, no data are present in the literature on albumin expression in mature adipocytes. In the present study, we found, for the first time, a clear albumin expression in human mature adipocytes. In addition, on the basis of our results, we can also reasonably affirm that differentiated adipocytes are probably able to synthesize albumin.

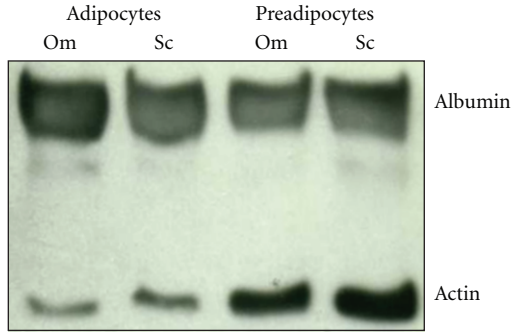

Figure 4: Representative immunoblot shows the albumin expression in Om and Sc adipocytes and preadipocytes isolated from a noninflamed control. Actin was used as loading control.

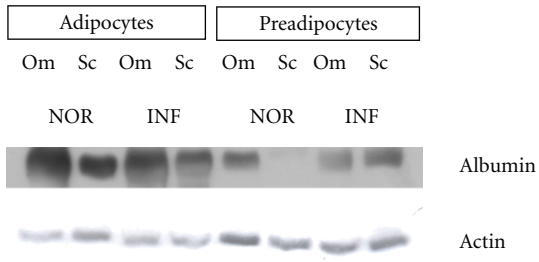

Figure 5: Representative immunoblot shows the albumin expression in Om and Sc adipocytes and preadipocytes isolated from both inflamed patients (INF) and noninflamed controls (NOR). Actin was used as loading control.

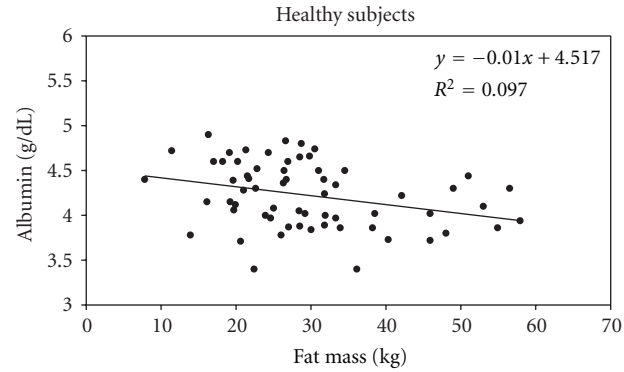

Figure 6: Negative relationship between Albumin circulating levels and Fat Mass. The Albumin circulating levels and Fat Mass were measured in 63 noninflamed patients. Correlation is significant at the 0.05 level (2-tailed). $R = -0.312$.

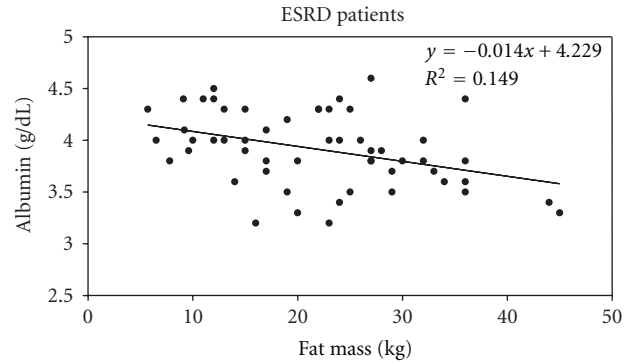

Figure 7: Negative relationship between Albumin circulating levels and Fat Mass. The Albumin circulating levels and Fat Mass were measured in 54 inflamed ESRD (End-Stage Renal Disease) patients. Correlation is significant at the 0.01 level (2-tailed). $R = -0.391$.

Albumin gene expression resulted significantly lower in the adipocytes than in the pre-adipocytes; in particular, it was 42 e 12 times lower in Om and Sc adipocyte, respectively, as compared with the pre-adipocyte (Figure 3). In this way, the omental pre-adipocyte represents the most active cell in albumin gene expression.

The presence of albumin in the pre-adipocyte is not a novelty. In fact, Yoo and Lee investigated the role of albumin in adipocyte differentiation, by using pre-adipocytes cell lines such as 3T3-L1 [26]. This is a developmental process by which undifferentiated precursor cells differentiate into mature adipocytes with coordinated changes in cell morphology and gene expression. They found that albumin gene expression was significantly increased at later stages of adipocyte differentiation process and its suppression significantly inhibited lipid droplet formation [26]. The author suggests that albumin could be necessary to stabilize lipid accumulation in mature adipocyte, probably through a direct interaction with fatty acids. However, it is important to underline that these experiments were performed on murine cell lines and their results cannot correspond to human cell data.

In a previous paper, we studied the involvement of adipose cells in patients with chronic inflammatory disease. We found that not only C-RP gene expression was activated in adipocyte cells, but both IL-6 and IL-6 receptors gene expressions were found to be higher in inflamed patients than in controls, either in subcutaneous or intra-abdominal adipose tissue [5].

At this regard, in the present study we hypothesized that adipocytes, similarly to hepatocytes, show a different albumin gene expression in inflamed and noninflamed patients, with a lower gene expression in inflamed ones. However, our results showed no significant difference in albumin gene expression between inflamed and noninflamed patients when analyzed by Real-Time PCR (Figure 2). On the other hand, Figure 2 shows also that albumin gene expression was significantly higher in intra-abdominal than in subcutaneous adipocytes (Figure 2).

Albumin presence, as protein, together with gene expression in adipocytes raises the hypothesis that adipose tissue contribute to circulating albumin levels, as well as it happens for IL-6 [3]. To verify this hypothesis we evaluated the relationship between serum albumin and fat mass, supposing that higher fat mass corresponds to higher circulating albumin levels. However, our results did not confirm this hypothesis, but we found a negative significant correlation between albumin and fat mass both in healthy noninflamed subjects and inflamed ESRD patients, in contrast with what

we expected (Figures 6 and 7). In other words, the higher the fat mass the lower was serum albumin concentration was. We suppose that higher fat mass leads to higher production of different proinflammatory cytokines, mainly IL-6, that can downregulate albumin, production in hepatocyte via endocrine way, independently of systemic inflammation. We also suppose that IL-6 produced by adipocyte could downregulate, via an autocrine and/or paracrine signaling, albumin gene expression and production in the adipocyte itself. This mechanism could explain both the lack of modulation of albumin gene expression by systemic inflammation in the adipocyte and the negative correlation between fat mass and albumin levels either in noninflamed subjects or in inflamed ESRD patients. On the contrary, this mechanism could not operate with inflammatory proteins, such as C-RP [5].

Despite the lack of albumin gene modulation by inflammation, in this study something really new was observed: the human adipocyte, once considered a simply depot cell, is now seen as a new and active cell, that in parallel with hepatocyte, is able to produce different proteins, such as C-RP and, as novelty, albumin.

Why adipocyte shows hepatocyte-like activity is still unknown. The more fascinating hypothesis states the existence of a continuum in adipose tissue cell population that goes from multipotent stem cell to more mature progenitor pools [6], passing through the pre-adipocyte. This hypothesis might explain why adipocyte and hepatocyte share the expression of some genes, such as albumin gene. As above mentioned, adipose-tissue-derived MSC (ADMSC) displayed the capacity to differentiate into numerous cell types (muscular, neuronal, bone, adipose cells) and, interestingly, into hepatocyte-like cells [13–15]. A study showed that ADMSC could be differentiated into functional hepatocyte-like cells by the treatment of cytokine mixtures *in vitro* [16], so to become a potential source to hepatocyte regeneration or liver cell transplantation [27]. In another work, the authors showed that the undifferentiated naïve ADMSC were also positive for albumin, G-6-P, and α-1-antitrypsin (AAT), which are all known to be predominantly expressed in adult liver cells [28].

However, we do suppose that other, more fascinating mechanisms, apart from sharing the same origin, may explain why, to date, adipocyte and hepatocyte produce a so important protein in our organism, like albumin.

In conclusion, this preliminary study highlights for the first time a new adipocyte activity, among the others already known; however further investigations are needed to confirm and explain our results.

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Clinical Study

Cytokines in Pericardial Effusion of Patients with Inflammatory Pericardial Disease

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Background. The role of inflammatory and angiogenic cytokines in patients with inflammatory pericardial effusion still remains uncertain. *Methods*. We assessed pericardial and serum levels of VEGF, bFGF, IL-1β and TNF-α by ELISA in patients with inflammatory pericardial effusion (PE) of autoreactive ($n = 22$) and viral ($n = 11$) origin, and for control in pericardial fluid (PF) and serum ($n = 26$) of patients with coronary artery disease (CAD) undergoing coronary artery bypass graft surgery. *Results*. VEGF levels were significantly higher in patients with autoreactive and viral PE than in patients with CAD in both PE ($P = 0,006$ for autoreactive and $P < 0,001$ for viral PE) and serum ($P < 0,001$ for autoreactive and $P < 0,001$ for viral PE). Pericardial bFGF levels were higher compared to serum levels in patients with inflammatory PE and patients with CAD ($P \leq 0,001$ for CAD; $P \leq 0,001$ for autoreactive PE; $P = 0,005$ for viral PE). Pericardial VEGF levels correlated positively with markers of pericardial inflammation, whereas pericardial bFGF levels showed a negative correlation. IL-1β and TNF-α were detectable only in few PE and serum samples. *Conclusions*. VEGF and bFGF levels in pericardial effusion are elevated in patients with inflammatory PE. It is thus possible that VEGF and bFGF participate in the pathogenesis of inflammatory pericardial disease.

1. Introduction

The management of pericardial effusion is an important clinical problem that remains challenging. However, the pathogenesis of pericardial fluid accumulation in different forms of pericardial disease is still not completely understood. Inflammatory reactions are a major cause of pericardial effusion. Studies on inflammatory pericardial effusions may elucidate the mechanisms of pericardial fluid formation and also the general mechanisms of pericardial inflammation. Cytokines are essential mediators of normal and pathologic immune and inflammatory responses. Particularly, inflammatory and angiogenic cytokines are involved in the pathogenesis of cardiovascular diseases, including atherosclerosis, myocardial infarction, myocarditis, and cardiomyopathy [1–3]. However, very few data exist on the activation pattern and pathophysiological role of inflammatory and angiogenic cytokines in pericardial diseases [4–6]. There are numerous studies on cytokines in pleural effusion [7–12] in contrast to the limited experience on pericardial cytokines in pericardial effusion. Cytokines, such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) are candidates for the induction of pericardial effusions because they have been implicated in the induction and amplification of inflammatory reactions in exudative effusions [7, 9, 10, 13–17]. Therefore we measured the concentrations of VEGF, bFGF, IL-1β, and TNF-α in pericardial fluid and serum from patients with inflammatory, nonmalignant pericardial effusion and patients without pericardial disease.

2. Materials and Methods

2.1. Study Population. The study population included 33 consecutive patients with inflammatory pericardial effusion (PE) who underwent subxiphoid, fluoroscopically guided pericardiocentesis, pericardioscopy, and peri-/epicardial

biopsy for therapeutic and/or diagnostic reasons after written informed consent [18]. For comparison, pericardial fluid (PF) from 26 patients was obtained and served during open-heart surgery for coronary artery disease together with a blood sample for each patient. None of the patients of the control group had a history of pericardial disease or evidence of pericardial effusion in echocardiography. The study was approved by the local ethics committee.

The etiologic diagnosis of inflammatory PE followed the criteria defined by the task force on pericardial diseases of the European Society of Cardiology [19]. In brief, the diagnosis of autoreactive inflammatory PE was based on the following criteria [19, 20]: (1) increased number of lymphocytes/mononuclear cells > 5000/mm^3 or the presence of antimyocardial antibodies in pericardial fluid; (2) inflammation in epicardial biopsies by ≥14 cells/mm^2; (3) exclusion of active viral infection in PE and epicardial biopsies (no virus isolation, negative PCR for major cardiotropic viruses, no IgM-titer against cardiotropic viruses in the PE); (4) exclusion of tuberculosis, Borrelia burgdorferi, Chlamydia pneumonia, and other bacterial infections by polymerase chain reaction (PCR) and/or cultures; (5) absent neoplastic infiltration in pericardial fluid and biopsy specimens; (6) exclusion of systemic, metabolic disorders and uremia. Viral inflammatory pericarditis was diagnosed by the presence of viral genome (parvovirus B19, influenza A/B, cytomegalovirus, enterovirus, adenovirus, herpes simplex virus, and Epstein Barr virus) detected by PCR in pericardial fluid and/or peri-/epicardial biopsies. For extraction of DNA/RNA from PE and peri-/epicardial biopsies, the QIAamp Blood Mini Kit and the QIAamp Tissue Kit (Qiagen, Hilden, Germany) were used. Conditions for PCR and primers have been described elsewhere [20].

2.2. Sampling of Pericardial Effusion, Pericardial Fluid, and Peri-/Epicardial Biopsies and Serum. In patients with inflammatory PE, pericardial effusion samples were obtained with pericardiocentesis. Pericardial fluid from patients with coronary artery disease (CAD) was obtained immediately after incision of the pericardium during coronary artery bypass graft surgery. Pericardiocentesis was performed in the cardiac catheterization laboratory using the subxiphoid route under local anaesthesia, with electrocardiographic and haemodynamic monitoring. After aspiration of pericardial fluid, a 0.038″ J-tip guidewire was introduced and its position checked in the lateral 90 degrees, the posterior-anterior, and the right anterior oblique views. The guide wire was finally exchanged for a 7 French pigtail catheter. Pericardial fluid was removed by manual suction. Pericardioscopy was performed in the same session as pericardiocentesis. After the evacuation of the pericardial effusion, the pericardial catheter was exchanged for a 16 French introductory sheath. A flexible endoscope (Karl Storz AF 1101 Bl) was introduced in the pericardial space and up to eight peri-/epicardial biopsies were taken under direct eye control through the working channel of the instrument.

PE samples were divided for laboratory analysis including basic biochemical and cell count parameters, cytology, bacterial cultures, and polymerase chain reaction (PCR) for identification of cardiotropic viruses (influenza virus A/B, Parvovirus B19, cytomegalovirus, enterovirus, adenovirus, human herpes virus 6, and Epstein Barr virus), as well as Borrelia burgdorferi, Chlamydia pneumonia, and Mycobacterium tuberculosis. For cytokine measurement all PE, pericardial fluid (PF), and serum samples were promptly aliquoted, transferred into chilled sterile tubes containing a proteinase inhibitor cocktail (Complete; Roche, Penzberg, Germany), and subsequently stored at −80°C until analysis.

The epi- and pericardial biopsies were fixed and processed in the usual manner, embedded in paraffin, cut into 4 mm serial sections by microtome, and then stained with haematoxylin-eosin for routine histology and Ziehl-Neelson stain for mycobacteria. Pathohistology examination was always performed by two independent experts.

For immunochemistry, biopsy samples were separately fixed and processed for identification and characterization of infiltrating cells with monoclonal antibodies, bound immunoglobulins (IgP, IgG, IgM and IgA), and complement fixation. The following markers on infiltrating cells were analyzed: CD2, CD3, CD4, CD8, CD19, CD 16, CD45Ro, T200, CD54, CD25, CD14, and CD11c.

2.3. Immunoassay for VEGF, bFGF, IL-1β, and TNF-α. The levels of cytokines have been successfully determined in pleural and pericardial effusion in previous investigations using an enzyme-linked immunosorbent assay [4, 7, 16, 21, 22]. In the present study, cytokine levels in PE and serum were measured blinded to any clinical information with a commercially available sandwich enzyme-linked immunosorbent assay (ELISA) from R&D Systems (Quantikine colorimetric sandwich ELISA, R&D Systems) according to the manufacturer's guidelines. In brief, after standard procedures, for each cytokine all samples were pipetted into the wells of the microtitre plates, specific horseradish peroxidase-linked polyclonal antibodies were added, and immunoreactive levels of the cytokine were determined. The detection limit for $VEGF_{165}$, bFGF, IL-1β, and TNF-α was 9 pg/mL, 3 pg/mL, 1 pg/mL, and 1,6 pg/mL, respectively. Both interassay and intra-assay coefficients of variation were <10% for $VEGF_{165}$, bFGF, IL-1β, and TNF-α.

2.4. Statistical Analysis. Results are given as mean ± standard deviation. Values below the detection limit were assumed to be zero for statistical analysis. All P values < 0.05 were considered statistically significant. For statistical analysis the software packages SigmaPlot version 11.0 were used. Comparison of VEGF levels between the three groups was performed by Kruskal-Wallis test. In case of significant differences between the groups, closed testing principle was applied to compare two groups by use of the Mann-Whitney U test. Correlation between variables was calculated using the Spearman test.

3. Results

According to the results of pericardial effusion and peri-/epicardial biopsy samples analysis, 22 (66%) patients had autoreactive inflammatory PE (12 females, 10 males,

Table 1: Demographic characteristics of study patients.

Variable	All patients ($n = 59$)	aPE ($n = 22$)	vPE ($n = 11$)	CAD ($n = 26$)	P value*
Men/women	39/20	10/12	8/3	21/5[#]	<0.05
Age (years)	66.7 ± 11.4	63.5 ± 11.6	58.7 ± 11.7	68.58 ± 8.3[‡]	<0.05
CAD	27 (46%)	1 (5%)	0	26 (100%)[#‡]	<0.001
Hypertension	42 (71%)	11 (50%)	5 (45%)	26 (100%)[#‡]	<0.001
Diabetes mellitus	12 (20%)	2 (4%)	0	10 (38%)[#‡]	<0.05
Hypercholesterolemia	44 (75%)	14 (64%)	6 (55%)	24 (92%)[#‡]	<0.05
Creatinine (mg/dL)	1.2 ± 1.24	0.88 ± 0.23	1.7 ± 2.5	1.29 ± 0.6[#]	<0.05
Hemoglobin (g/L)	135 ± 18.77	138.86 ± 15.6	124.36±22.72	137 ± 18.21	NS
Leukocytes (G/L)	8.53 ± 2.81	8.62 ± 3.11	9.78 ± 2.99	7.61 ± 1.98	NS
Protein (g/dL)	69.86 ± 7.98	71.57 ± 6.68[‡]	63.18 ± 9.11	72.06 ± 6.63[‡]	<0.05
C-reactive protein (mg/L)	21.88 ± 46.61	24.76 ± 48.66	43.46 ± 66.13	3.25 ± 4.47	NS

Values are mean ± SD or numbers of patients (percentage).
*Analysis of variance/chi-square or Fischer exact test.
[#]$P < 0.05$ versus aPE; [‡]$P < 0.05$ versus vPE.
In 9 patients with CAD and 1 patient with aPE no biochemical data were available.
aPE: autoreactive pericardial effusion; vPE: viral pericardial effusion; CAD: coronary artery disease; NS: not significant.

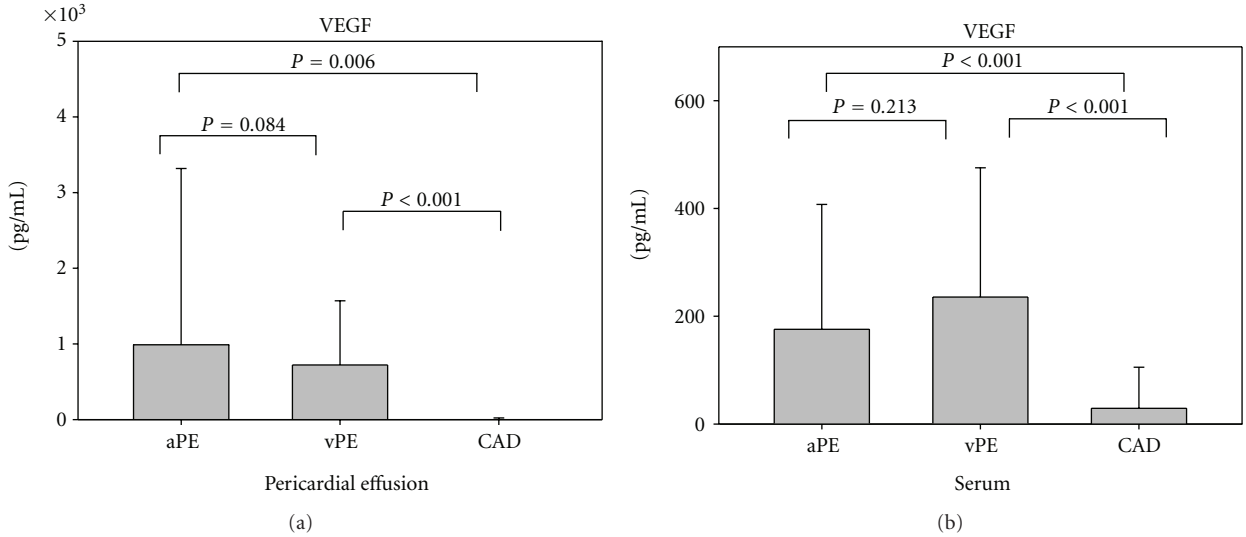

Figure 1: VEGF levels measured in pericardial effusion/pericardial fluid (a) and serum (b) from patients with autoreactive pericardial effusion, viral pericardial effusion, and coronary artery disease.
aPE: autoreactive pericardial effusion; vPE: viral pericardial effusion; CAD: coronary artery disease.

63,5 ± 11,6 years), and 11 (33%) patients had viral inflammatory PE (3 females, 8 males, 58,7 ± 11,7 years). For comparison, 26 patients with CAD (5 females, 21 males, 68,58 ± 8,3 years) were included in this investigation (Table 1).

VEGF was measurable in 10 (45%) PE and 18 (82%) blood samples from patients with autoreactive PE and in 9 (82%) PE and 8 (73%) blood samples from patients with viral PE. In CAD patients VEGF was detected only in 3 (12%) PF and 4 (15%) blood samples.

Pericardial VEGF levels in autoreactive and viral pericardial effusion were significantly higher compared to VEGF in PF ($P = 0{,}006$ and $P < 0{,}001$, resp.) (Figure 1(a)). However, pericardial VEGF levels did not differ between autoreactive and viral PE ($P = 0{,}084$). In serum, VEGF was significantly higher in patients with autoreactive and viral PE compared to patients with CAD patients ($P < 0{,}001$ and $P < 0{,}001$, resp.). As in PE, serum VEGF levels did not differ significantly between patients with autoreactive and viral PE ($P = 0{,}213$) (Figure 1(b)). Although statistical significance was not reached, pericardial VEGF levels were higher than matched serum levels in 6 out of 22 (27%) patients with autoreactive PE and in 6 out of 11 (55%) patients with viral PE, ($P = 0{,}07$ for autoreactive PE; $P = 0{,}279$ for viral PE).

Basic FGF was detectable in 20 (91%) PE and 3 (14%) serum samples from patients with autoreactive PE, 10 (91%) PE samples and 1 (9%) serum sample from patients with

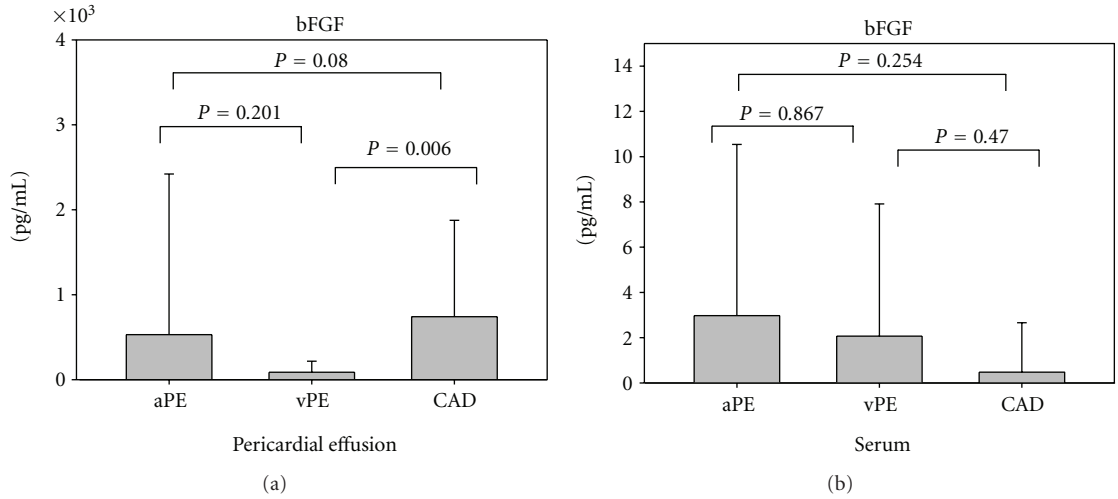

Figure 2: Basic FGF levels measured in pericardial effusion/pericardial fluid (a) and serum (b) from patients with autoreactive pericardial effusion, viral pericardial effusion, and coronary artery disease.
aPE: autoreactive pericardial effusion; vPE: viral pericardial effusion; CAD: coronary artery disease.

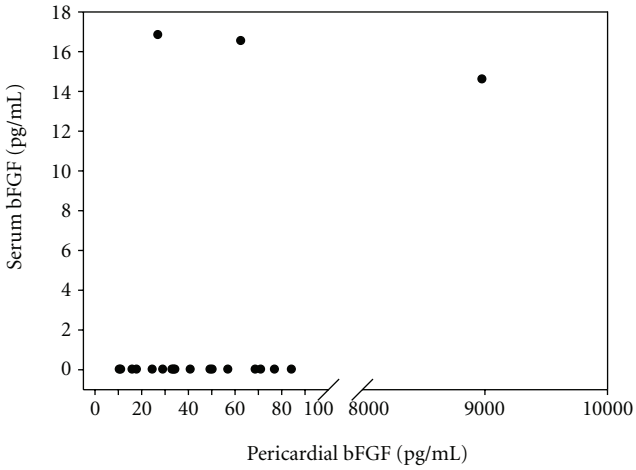

Figure 3: Pericardial and corresponding serum bFGF levels in patients with inflammatory pericardial effusion.

viral PE, and 25 (96%) PF samples and 1 (3,8%) serum sample from patients with CAD. Pericardial bFGF levels were highest in patients with CAD, although statistical significance was only reached between CAD patients and patients with viral PE (P = 0,006) (Figure 2(a)). In serum, bFGF was detectable only in 3 patients with autoreactive PE, 1 patient with viral PE and 1 patient with CAD (Figure 2(b)). Pericardial bFGF levels were higher compared to serum levels in patients with CAD and patients with inflammatory PE (Figure 3) ($P \leq$0,001 for CAD; $P \leq$0,001 for autoreactive PE; P = 0,005 for viral PE).

IL-1β was detectable only in few PE (1 patient with viral PE and 1 patient with CAD) and serum samples (1 patient with viral PE). Obtained pericardial and serum levels did not differ between autoreactive PE, viral PE, and CAD.

TNF-α was detectable in PE of a smaller proportion of patients with inflammatory PE (2 patients with autoreactive PE and 3 patients with viral PE) and in none of the patients with CAD. In serum, none of the patients with inflammatory PE or CAD had detectable TNF-α levels.

Table 2: Pericardial levels of related biochemical parameters in autoreactive and viral PE.

Variable	Autoreactive PE	Viral PE	P value*
LDH (U/L)	405.1 ± 761.35	902.36 ± 1008.77	0.08
Protein (g/dL)	37.05 ± 13,59	36.55 ± 10.9	0.92
Glucose (mg/dL)	95.5 ± 14.15	97.36 ± 21.73	0.77
Leukocytes (G/L)	1.47 ± 3.33	1.46 ± 1.24	0.15

Values are mean ± SD.
*Analysis of variance/t-test.
In 2 patients with autoreactive PE no biochemical data were available.
PE: pericardial effusion.

Related biochemical parameters and leukocytes counts in PE from patients with autoreactive and viral PE are depicted in Table 2. Pericardial VEGF levels correlated positively with markers of pericardial inflammation (Table 3). In contrast, a negative correlation between pericardial bFGF and markers of pericardial inflammation was observed (Table 3).

4. Discussion

The present study investigated for the first time the pericardial and serum concentrations of VEGF, bFGF, IL-1β, and TNF-α in pericardial effusion and serum from patients with inflammatory pericardial effusion and compared them with pericardial fluid and serum levels of patients with CAD and no history or evidence of pericardial disease. Analysis of cytokines in pericardial effusion provides a modality to elucidate the pathophysiology of pericardial disease and may help to identify the etiology in patients with PE [19, 23]. However, studies related to cytokine analysis in pericardial effusion are rare. Initial investigations on

Table 3: Correlation between VEGF and bFGF and markers of pericardial inflammation in patients with inflammatory pericardial effusion.

Variable		VEGF	bFGF
LDH (U/L) in PE	r	0.655	−0.542
	P	<0.001	0.002
Leukocytes (G/L) in PE	r	0.543	−0.443
	P	0.003	0.02

VEGF: vascular endothelial growth factor; bFGF: basic fibroblast growth factor; PE: pericardial effusion; r, Spearman coefficient of correlation; P: P-value.

pericardial cytokines included only case reports [5, 6]. In contrast, a systematic analysis of pericardial interleukin-6, interleukin-8, and interferon-γ investigated 101 patients with pericardial effusion [4]. However, intrapericardial VEGF, bFGF, IL-1β, and TNF-α levels in inflammatory PE have not been studied before.

In contrast to the limited experience on cytokines in inflammatory pericardial effusion, numerous investigations on cytokines in pleural effusion exist. Some of the findings could be possibly extrapolated for pericardial effusion.

Pleural (and pericardial) inflammation is typically accompanied by enhanced vascular permeability and both are essential for the pathogenesis of inflammatory effusions leading to increased fluid formation. Several studies confirm that VEGF, which has potent angiogenic, mitogenic, and permeability-inducing properties could be implicated in the pathogenesis of exudative pleural effusions. This notion is largely based on the findings that the levels of VEGF were consistently higher in exudative than in transudative pleural effusions [7, 16, 21, 24] and that pleural VEGF levels correlate with markers of pleural inflammation [16, 22]. In line with this data, we found higher pericardial VEGF levels in exudative, inflammatory PE than in PF from patients with CAD. Moreover, VEGF levels in inflammatory PE correlated positively with markers of pericardial inflammation (leukocytes and LDH in pericardial effusion).

A recent study by Wörnle et al. also demonstrated that viral infections lead to an increase in VEGF synthesis from mesothelial cells [12]. According to the authors mesothelial VEGF synthesis after activation of viral receptors could represent the link between viral infections and formation of pleural effusion. Further studies are warranted to investigate whether these findings also apply for viral pericardial effusion.

The implication of bFGF in the pathogenesis of exudative effusions has been less extensively studied. Data from Ruiz et al. [16] have suggested that bFGF participates in the pathogenesis of exudative pleural effusions and Strizzi et al. [25] have demonstrated a larger contribution of bFGF in benign than malignant effusions. However, the study by Ishimoto et al. [26] questioned the implication of bFGF in the formation of pleural effusion. In our series, pericardial bFGF levels were regularly elevated in patients with inflammatory PE.

Inflammatory cells and activated endothelium can synthesize and release bFGF [17, 27–29]. The inflammatory reaction itself may also cause endothelial cell damage, which results in increased bFGF production and release [30]. Basic FGF may amplify the inflammatory reaction by recruiting inflammatory cells [31, 32]. Furthermore, bFGF can induce vascular permeability directly and indirectly by upregulating VEGF [33]. However, bFGF was negatively correlated with markers of pericardial inflammation (leukocytes and LDH). One possible explanation may be that long-lasting exposure to bFGF reduces leukocyte migration and response to various chemokines [31, 34, 35] after the initial amplification of the inflammatory response. Further study is required to corroborate this finding.

In inflammatory pericardial effusion, the presence of cytokines within the pericardial cavity may be explained by intrapericardial production or diffusion from systemic circulation. Therefore, we compared pericardial and serum levels of the VEGF, bFGF, IL-1β, and TNF-α in order to examine whether local pericardial production is the main source of these cytokines. We observed that pericardial bFGF was significantly higher compared to corresponding serum bFGF, whereas pericardial VEGF tended to be higher than serum levels, but the difference did not reach statistic significance. These findings may reflect preferential local pericardial production of bFGF, whereas our results seem less clear for VEGF. Within the pericardial cavity, cytokines are likely to originate from multiple cellular sources. Infiltrating leukocytes, activated mesothelial cells, or epicardial adipose tissue may be involved. [17, 27, 28, 36]. The positive correlation between pericardial VEGF and pericardial leukocytes suggests that pericardial leukocytes might be one source of VEGF. Several investigations have also shown that pleural mesothelial cells are metabolically active and possess the capacity to produce various cytokines [37–39]. However, so far no study addresses the role and direct participation of pericardial mesothelial cells in the pathogenesis of pericardial inflammation.

In addition, we observed markedly elevated bFGF levels in PF from patients with CAD. In agreement with our results, high bFGF levels in PF of patients with ischemic heart disease have been reported before [1]. It has been suggested that bFGF in PF may accelerate the growth of human vascular smooth cells, induce angiogenesis, and mediate collateral development [1, 40]. Intrapericardial administration of bFGF promoted angiogenesis and reduced infarction size in dogs with acute myocardial infarction [41].

IL-1β and TNF-α were detectable only in a small proportion of patients with inflammatory PE. These results suggest that these cytokines play a minor part in inflammatory PE.

In conclusion, this study provides new data regarding the role of cytokines in the pathophysiology of inflammatory pericardial effusions. Elevated VEGF and bFGF levels in inflammatory pericardial effusions indicate that these growth factors might be involved in the pathogenesis of inflammatory pericardial effusions.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgments

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Review Article

Pentraxin 3(PTX 3): An Endogenous Modulator of the Inflammatory Response

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Inflammatory or anti-inflammatory? That is the question as far as the acute-phase response and its mediators, the pentraxins, are concerned. Only some ten years ago, the classical or short pentraxin C-reactive protein and the newly discovered long pentraxin PTX3 were considered to exert most of the detrimental effects of acute inflammation, whether microbial or sterile in origin. However, accumulating evidence suggests an at least dichotomous, context-dependent outcome attributable to the pentraxins, if not a straightforward anti-inflammatory nature of the acute-phase response. This paper is focused on the inherent effects of pentraxin 3 in inflammatory responses, mainly in coronary artery disease and in *Aspergillus fumigatus* infection. Both are examples of inflammatory reactions in which PTX3 is substantially involved; the former sterile, the latter infectious in origin. Apart from different inducing noxae, similarities in the pathogenesis of the two are striking. All the same, the introductory question still persists: is the ultimate impact of PTX3 in these conditions inflammatory or anti-inflammatory, paradoxical as the latter might appear? We try to provide an answer such as it emerges in the light of recent findings.

1. Danger Recognition

The innate immune system provides the first line of defense against microorganisms with which humans are daily contacted. The encounter with the microbial world occurs either on the body's external surface, that is, on the skin, or on its internal (mucosal) surfaces in the airways and in the gastrointestinal tract. The vast majority of these steady-contact microbes are not inherently pathogenic. Nevertheless, their eventual threat to the host may vary considerably according to both external/environmental and to internal conditions, the latter being reflected by the ability of the host to mount an appropriate immune response. Whenever needed, this response must go on to involve the adaptive immune reaction(s). Basically, the same holds true for virtually pathogenic microorganisms. An infection may be brought right under control if confronted with an immunologically competent host. Actually, the net result of pathogen-host interaction, that is, whether health is maintained or disease develops, relies on the balance between pathogen virulence and the capacity of the individual immune response. The innate and adaptive immune reactions form a continuum of closely interrelated steps with innumerable positive and negative feedback loops. Therefore, they should be viewed as two sides of the same coin [1].

To initiate a defense reaction, first of all, a microorganism must be sensed and recognized by the immune system as potentially harmful. It has been established that microorganisms are not recognized in their individual complexity. Instead, several sets of highly conserved molecular moieties which are shared by large groups of microorganisms are implicated in the process of being recognized. These molecular motifs, collectively referred to as *alarmins* or *pathogen-associated molecular patterns* (PAMPs), are committed to

elicit a *danger signal* within the immune system. Importantly, closely resembling or even identical molecular structures are present in the host's own cells. Under normal conditions, these molecules are well hidden inside the cellular cytoplasm, far beyond the reach of the recognition machinery. In cells undergoing apoptotic or necrotic death, the hallmark of sepsis or ischemia-reperfusion injury, PAMPs/alarmins are promptly exposed on the surface membrane of the host's cells or even released into the extracellular space [2, 3]. Another way to exhibit own PAMPs/alarmins, albeit in a slow, gradual process, occurs in low-grade inflammation. Examples thereof are atherogenesis or rheumatoid arthritis. Inflammatory diseases which develop in the absence of microorganisms are called *sterile inflammation* [4].

The constituents of the immune system which are involved in the process of recognizing PAMPs, either of microbial origin in bacteremia/sepsis or modified self-structures in sterile inflammation, are represented by germ line-encoded receptors known as *pattern recognition molecules* (PRMs). The occurrence of PRMs is not confined to canonical components of the immune system, but other cells are also involved. From the viewpoint of cardiology/cardiac surgery, endothelial cells play a prominent role [5].

Within the human body, pattern recognition molecules/receptors are present either as *cell-associated-* or as *fluid-phase molecules.* The former are localized in most tissues, whereas the latter are distributed in the liquid compartment, that is, mainly, but not exclusively, in the blood. Cell-associated receptors are made up by endocytic/scavenger receptors, signalling receptors (e.g., toll-like receptors), and nucleotide-binding oligomerization domain- (NOD-)like receptors [6]. The fluid-phase molecules represent evolutionary ancestors of antigen-specific antibodies. This heterogeneous group of molecules consists of three clearly defined subgroups, that is, the collectins, the ficolins, and the pentraxins. All of them are implicated in complement activation, pathogen opsonisation, and/or self versus modified-self versus non-self discrimination [7].

2. A Brief Glance at the Pentraxin Superfamily

The pentraxins form a superfamily of multifunctional proteins which have been conserved in phylogeny from arachnids to mammals. The pentraxin superfamily is distinguished by the presence in their C(carboxy)-terminal region of a ~200 amino acid domain containing a highly conserved motif of 8-amino-acid sequence, which has been named the *pentraxin signature* (HxCxS/TWxS, where x is any amino acid). Based on the primary structure of the protomer, the pentraxins branch off into two groups: the short constituents and their long counterparts [8].

Short pentraxins are about 25-kDa proteins sharing a common structural organization of five or ten subunits that are assembled in pentameric radial symmetry. The classical short pentraxins C-reactive protein (CRP) and serum amyloid P component (SAP) are acute-phase proteins in humans and in mice, respectively. They are manufactured in the liver under the guidance of inflammatory cytokines, most prominently of interleukin (IL)-6 and, to a lesser degree, of IL-1β. CRP is the major acute-phase reactant in humans. Its levels are scarcely detectable in plasma of healthy persons, in whom CRP concentrations do not exceed 3 mg/L (3 μg/mL). However, CRP plasma concentrations increase as much as 1000-fold under inflammatory conditions, irrespective whether of bacterial (sepsis) or sterile (sepsis-like) origin. Classically, the latter develops in patients after trauma and surgical operations or in patients with acute coronary syndromes (unstable angina pectoris/acute myocardial infarction). CRP and SAP bind their respective ligands in a calcium-dependent manner. Both of them play important roles in innate resistance to microbes and in scavenging pathogenic cellular debris including extracellular matrix components [9]. Pentraxin 3(PTX3) was identified in the early 1990s in human endothelial cells and fibroblasts as a TNF-α- or IL-1β-inducible mRNA and protein, respectively. IL-6 does not deal with PTX3 production. Despite manifest similarities of action, PTX3 differs from its short associates in many basic aspects, such as gene organization, chromosomal localization, cellular sources, inducing stimuli and the recognized ligands [10].

3. Genetic Aspects of Pentraxin 3

The human *ptx3* gene is localized on the chromosome 3q band 25. It is composed of three exons, the first two of which encode for the signal peptide and the N-terminal domain (amino acids 18–179), respectively. The third exon encodes for the C-terminal domain featuring the pentraxin signature (amino acids 179–381) [11]. Immature myeloid dendritic cells were supposed to be the prevailing cellular population capable to produce PTX3. However, stimulus-induced PTX3 has been detected in other cellular populations, such as the monocytes/macrophages, smooth muscle cells, kidney epithelial cells, synovial cells, chondrocytes, adipocytes, alveolar epithelial cells, glial cells, fibroblasts, and, of course, in endothelial cells [12]. PTX3 promoters contain enhancer-binding elements which, during proteosynthesis, fine-tune final impact of PTX3 on its target structures. The most prominent ones are activator protein-1 (AP-1), nuclear factor-kappa B (NF-κB), and selective promoter factor 1 (SP1) [13]. Briefly, AP-1 enhances basal transcription of PTX3, whereas the NF-κB binding site is operative in the response to inflammatory cytokines TNF-α and/or IL-1β. Tissue-specifically, the above-mentioned transcription factors are complemented, in their proteosynthesis-modulating activities, by enzymatic biochemical pathways. *In lung epithelial cells* challenging acute inflammation, PTX3 mRNA as such is induced by TNF-α; nevertheless, PTX3 protein generation itself does not require consequent NF-κB transcription. Instead, PTX3 is manufactured by way of the c-Jun N-terminal kinase pathway [14]. *In endothelial cells*, expression of PTX3 is readily induced by TNF-α and IL-1β. Thereafter, an acute cellular alteration sets in, in which the endothelial cell is converted from a quiescent, anti-inflammatory phenotype, to a procoagulant and proinflammatory cellular surface. This is the case in sepsis/infection or in acute myocardial infarction. On the other

hand, endothelial cells are implicated in low-grade sterile inflammation that underlies atherosclerosis and small vessel vasculitides. A counter-regulatory pathway which abrogates unwanted inflammation is carried out by high-density lipoprotein 3 (HDL3) subfraction. This HDL3-controled PTX3 production evades classical regulatory mechanisms in that it relies on the activation of the PI3K/Akt pathway via G-coupled lysosphingolipid receptors. The lysosphingolipid receptor S1P, which is the one responsible for this alternative PTX3 synthesis, is a protein particle carried by HDL3. In human endothelial cells, therefore, PTX3 accomplishes dual outcome according to the respective activators: (i) an inflammatory effect induced by TNF-α and/or IL-1β or (ii) an anti-inflammatory effect induced by S1P/HDL3. The latter is further translated into increased NO-dependent vasorelaxation, endothelial cell antiapoptotic effects, and increased TGF-β expression with ensuing atheroprotective modulation [15]. PTX3 generation is also regulated in a cell-dependent manner by glucocorticoid hormones (GHs). GHs support the production of PTX3 in *fibroblasts* and *endothelial cells*, whereas they suppress PTX3 production by haematopoietic cells (*dendritic cells* and *macrophages*). Accordingly, intravenous administration of a glucocorticoid hormone increases blood levels of PTX3 [16].

4. Pentraxin 3 Storage in Neutrophils

Apart from the generation of PTX3 in a response to inflammatory stimuli, there is also a constitutive, ready-made form of PTX3 which is stored in specific (lactoferrin$^+$) granules of neutrophils. After these cells have been activated by appropriate stimuli, this preformed amount of PTX3 is released into the extracellular space. This is a virtually mature, that is, glycosylated form of monomeric PTX3 which, once occurring extracellularly, assembles into multimers of five subunits. The PTX3 glycosidic moiety presents a complex of bi-, tri-, and tetra-antennary sialylated structures, the relative number of which undergoes changes reflecting the inflammatory conditions in which actual PTX3 protein has been generated. Glycosylation of PTX3 seemingly functions as a fine-tuning mechanism of the mature protein. Thus, neutrophils represent a ready-to-use reservoir of PTX3 guaranteeing its early release and early activity in acute inflammation [17]. Under normal steady-state conditions, circulating neutrophils exhibit a short half-life terminated by apoptotic death, which serves to protect the host from any undue damage inflicted by these cells. Whenever inflammation is evoked, those stimuli which account for early PTX3 release delay concomitantly neutrophil apoptotic death. This is a natural feedback mechanism maintaining neutrophil numbers within required limits; otherwise, the host would incur premature neutrophil exhaustion leading to attenuation of inflammation. Delayed neutrophil apoptosis is not a selective mechanism. Prolonged neutrophil life-span is needed to support the host protection against infection/sepsis. However, the same scenario, albeit occurring in a slow step-by-step manner, is set in motion in chronic sterile inflammation in which increased numbers of activated neutrophils are harmful to the host. Atherosclerosis may serve as an example thereof, although participation of neutrophils in this process was long underestimated [18].

Upon inflammatory activation, neutrophils release about 25% of their actual PTX3 content. Part of the extruded protein remains associated with the parent cell by way of neutrophil extracellular traps (NETs). This is a chromatin material assembled with nuclear proteins which has been set free concomitantly with PTX. Localization of PTX3 in neutrophil extracellular traps contributes to the generation of an antimicrobial microenvironment which augments local capacity to trap and kill microbes. Given the abundance of neutrophils both in the circulation and in the inflammatory foci, these cells whose life-span has been prolonged into the bargain constitute the main source of PTX3 right after the onset of infection or in acute sterile inflammation, such as myocardial infarction. Neutrophil-released PTX3 is functionally competent well before *de novo* synthesis by other cells delivers supplemental amounts of the protein. At the same time, neutrophil-derived PTX3 accounts for another feedback mechanism which restricts excess transmigration of activated neutrophils into the host's tissues, thus dampening unwanted dissemination of inflammatory reactions [19].

5. Regulation of Pentraxin 3 Production

Activation stimuli which control PTX3 synthesis and release can be enumerated as follows: (i) proinflammatory cytokines (IL-1β, TNF-α), (ii) TLR agonists, namely lipopolysaccharide (LPS), (iii) distinct microbial moieties (OmpA, lipoarabinomannans), and/or (iv) some intact microorganisms. Taking into account the complexity of the inflammatory/immune reactions, more than one stimulus is usually employed to set off PTX3 production. To initiate the immune response, cell-expressed receptors tightly cooperate with their fluid-phase counterparts after they have recognized hostile microorganisms (*non-self*) via the latter's PAMPs. Essentially, the same scenario proceeds in cases of sterile inflammation, after the recognition machinery has discriminated own modified or damaged cell structures (*altered self*) [20]. To give an example, macrophage scavenger receptor CD36 mediates binding and internalization of Gram-positive bacteria by cooperating with toll-like receptors TLR2 and TLR6, thereafter initiating production of proinflammatory cytokines TNF-α, IL-1β, IFN-γ, and many others. Concurrently, production of both short and long pentraxins CRP and PTX3 by their paternal cells may be started off. In contrast to the current paradigm which claims that cytokines considered proinflammatory should be activated to start the inflammatory/innate immunity response, IFN-γ downregulates PTX3 production in dendritic cells and in monocyte-macrophage cell lines. To achieve this, IFN-γ decreases PTX3 mRNA transcription and reduces PTX3 transcript stability, respectively [21]. On top of it, LPS-induced PTX3 expression is downregulated by (i) interleukin-4, (ii) 1α,25-dihydroxivitamin D3, and (iii) prostaglandin E2 [22]. Additionally, PTX3 by itself does not bind to (i) lipopolysaccharide (LPS), (ii) lipoteichoic acid (LTA), (iii) enterotoxins

A, and B, (iv) exotoxin A and (v) N-acethylmuramyl-L-alanyl-D-isoglutamine (MDP). Interleukin-(IL-)10, a most prominent anti-inflammatory cytokine, enhances LPS-induced PTX3 production, however, paradoxically it might appear. Due to the impact of IL-10 on dampening inflammation and supporting wound healing, this fact implies that PTX3/IL-10 would play an imortant role in tissue repair [23].

6. Pentraxin 3 in Cardiovascular Diseases

Important pieces of knowledge regarding PTX3 behavior in cardiovascular diseases, such as atherosclerosis and acute myocardial infarction (AMI), have been gained in the last ten years. As early as in 2000, Peri and coworkers published in *Circulation* their results from a cohort of 37 patients who had presented with an AMI. The authors showed that in these patients, plasma levels of PTX3 peaked as early as ~6 hours after the onset of chest pain. Thus, maximal concentration of PTX3 was available much earlier than that of CRP which is achieved after ~48 hours. In AMI patients, individual levels of PTX3 were attained independently of the extent of myocardial necrosis or incident heart failure assessed by the Killip class [24]. Four years later, these authors extended their original findings by adding that in AMI patients, PTX3 supplied the most powerful information regarding three-month mortality after the index event. In their study, prognostic value of PTX3 was superior to that provided by CRP, CK, TnT, and NT-proBNP [25]. Additional studies introduced PTX3 as a prognostic biomarker in heart failure, both acute and chronic, which was unrelated to an AMI [26, 27]. In the cardiovascular health study, PTX3 has been established to associate with the incidence of CAD and all-cause mortality in CAD patients, independently of CRP and other classical risk factors [28]. In our own study dealing with plasma PTX3 changes in patients undergoing coronary artery bypass grafting, we expectedly documented hundred-fold elevations of CRP in all patients, none of whom suffered any peri- or postoperative complication. However, PTX3 did not exceed the level of 2 ng/mL in any blood sample collected during or up to the 7th day after the operation, this respective value has been set as normal for healthy humans [29]. Studies examining the metabolic syndrome, a prominent risk factor of CAD, do also point out to discordant comportment of CRP and PTX3. Whereas CRP appears to correlate *positively* with (i) body weight, (ii) body-mass index, (iii) waist circumference, (iv) fasting plasma glucose, and (v) plasma IL-6 levels and *negatively* with (i) HDL cholesterol and (ii) adiponectin, PTX3 displays the opposite trends. It can be soundly assumed that the functions of PTX3 and CRP complement or even overlap in some situations, wheres in others they diverge substantially. There are even first hints that PTX3 might play an as yet unrecognized protective role in atherosclerosis and its complications [30].

7. Pentraxin 3 in Atherosclerosis

Studies examining PTX3 in the process of atherogenesis found this long pentraxin to be expressed in human vascular smooth muscle cells via atherogenic lipoproteins [31], to upregulate tissue factor expression both in human endothelial cells [32] and in activated monocytes [33], and to occur extensively in advanced atherosclerotic plaques. Therein, the source of PTX3 is not confined to macrophages and surviving endothelial cells [34], but, importantly, PTX3 is also present in infiltrating neutrophils [35], a cell population increasingly recognized as being active in the initial phase of the disease as well as in its complications [36, 37]. Clinically, PTX3 has been found to operate actively in unstable angina pectoris [38, 39] and in restenosis after coronary artery stent introduction; however, without identifying its source(s) in these conditions [40]. Furthermore, PTX3 displays a predilection to segments of vascular or synovial inflammation in rheumatic diseases, the pathogenesis of which is supposed to have much in common with that of atherosclerosis [41]. As it is, the inaugural question stands out with an intriguing acuity: is pentraxin 3 an inflammatory/harmful or an anti-inflammatory/benefitial mediator?

This question has been partly resolved with the accession of genetically modified mice in whom the PTX3 gene has been silenced (*ptx3*$^{-/-}$ mice). These animals do not produce any traces of PTX3 protein. When cross-bred with apolipoprotein E gene-deficient (*apoe* $^{-/-}$) mice, which is a murine phenotype closely recapitulating the development of human atherosclerosis, mouse strains which result from these combinations (PTX3$^{+/+}$ or PTX3$^{+/-}$ ApoE$^{-/-}$ versus PTX3$^{-/-}$ ApoE$^{-/-}$) yielded exciting insights into the involvement of PTX3 in atherogenesis. During the course of aging, PTX3$^{-/-}$ apoE$^{-/-}$ mice develop, in line with expectations, increasing atherosclerosis as a function of time. When fed Western-type diet, these mice display an even increased extent of atherosclerosis. In both settings, that is, normal chow versus Western-diet-fed mice, PTX3 amounts correlated positively with the atherosclerotic burden. By contrast, in PTX3$^{+/+}$ apoE$^{-/-}$ double knockout mice, the atherosclerotic affliction is unexpectedly enhanced instead of being diminished, which should have been the case if PTX3 was an unequivocal atherogenic mediator. Further studies specified that in atherosclerosis-prone mice, total absence of PTX3 was pronounced in the vascular wall by increased upregulation of (i) proinflammatory mediators such as the chemokines (e.g., CCL2, CCL3, CCL5, CCL7, CCL8) or cytokines (e.g., IL-4, IL-6, TNF-α), (ii) transcription factors such as NF-κB and the related protein Irak1, Fos, Jun, GATA3, GATA4, Egr2, Egr3, (iii) key mediators of the inflammatory response (e.g., TLR-2, TLR-4), and/or (iv) adhesion molecules responsible for the onset of endothelial dysfunction: intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), endothelial leukocyte adhesion molecule-1 (E-selectin), platelet/endothelial cell adhesion molecule (PECAM) [42].

8. Pentraxin 3 in Myocardial Infarction and in Ischemia/Reperfusion Injury

Alberto Mantovani's team furthermore evidenced that in genetically modified mice, PTX3 plays a protective role in acute

myocardial infarction, notably in the reperfusion phase. It must be pointed out here that PTX3 is not expressed in the heart under resting conditions, either murine or human. During reperfusion of a previously ischemic myocardium (or, practically, any tissue), an inflammatory response is elicited by explosive production of reactive oxygen species. In the course of reperfusion, an injury develops which is characterized by leukocyte activation and recruitment thereof into the reperfused area; by endothelial dysfunction which paves the way to these cells'apoptotic or necrotic death and/or by cardiomyocyte cell death which occurs as a result of blood flow defects (the "no-reflow" phenomenon). In a model of coronary artery ligation/reperfusion, $PTX3^{-/-}$ mice exhibit more extensive myocardial damage compared to their wild-type littermates. Specifically, reperfusion injury is manifest as (i) increased activated neutrophil and monocyte/macrophage infiltration to the damaged tissue, (ii) decreased number of tissue capillaries including extension of the no-reflow area by endothelial swelling which results in an obstruction of the remaining vessels, and/or (iii) increased number of apoptotic cardiomyocytes caused either by microcirculatory drop-outs, or inflicted to the cardiomyocytes by activated complement. Conversely, administration of exogenous PTX3 to PTX3-deficient mice dampens this injury down to the level experienced by their wild-type littermates. Administration of surplus PTX3 protein seems to confer a supplemental protection not only to the deficient, but also to normal, that is, $PTX3^{+/+}$ mice [43]. As far as humans are concerned, the situation requires much of further research.

9. Interaction of Pentraxin 3 with Platelets in Acute Myocardial Infarction

Activated platelets cause much of tissue damage in myocardial infarction. Hence, they are the predominant target cells of PTX3. Upon extrusion into the extracellular space, neutrophil-originating PTX3 binds to adjacent platelets which, thereupon, lose much of their robust inflammatory armamentarium. PTX3-stained platelets are thereafter resistant to the formation of platelet-platelet homoaggregates or to platelet-neutrophil or platelet-monocyte heteroaggregates. Furthermore, PTX3 broadly impacts the adhesion molecule P-selectin, which is known to play distinct roles in atherogenesis. PTX3 brings about downregulation of P-selectin-dependent neutrophil recruitment to inflammatory sites and of P-selectin-induced cellular heteroaggregate formation. Hence, decreased numbers of microaggregates are present that would impair blood flow in the microcirculation, not to speak of their additional noxious potential. In acute coronary syndromes, the overall procoagulant state is increased, including excess tissue factor formation by different cell types which makes the blood more "sticky" [44].

Intervention of either endogenous or exogenous PTX3 mitigates the development of the "no-reflow" fenomenon, that is, sustained tissue hypoxia/anoxia despite successful recanalization of large arteries. The jeopardized tissue is imbibed, irrespective of its extent, by a dense infiltrate which is composed largely of granulocytes and macrophages. The same applies to the early necrotic scar before its fibrotic transformation. PTX3 can be detected in most of the infiltrating macrophages and remaining endothelial cells, whereas neutrophils are scarcely found by this time point. If so, a few granulocytes are localized mainly in the center of the lesion, whereas in the outer parts, PTX3 is found only extracellularly. Cardiomyocytes are constantly devoid of PTX3 [45]. On the subcellular level, activities of PTX3 impact the transcription factor NF-κB and its modifying effects on proteosynthesis, since the beneficial outcome is lost in IL-1RI- or MyD88-deficient mice. MyD88 is a cellular adaptor protein downstream of the receptors for TLR and IL-1R. MyD88- or IL-1R-deficient mice are unable to upregulate PTX3 expression and in cases of acute coronary events, these artificial animals behave like those who are entirely lacking the long pentraxin (PTX3-deficient or $PTX3^{-/-}$ mice). Thus, the IL-1R-MyD88 pathway plays a dominant role in ptx3 mRNA induction in heart ischemia. In MyD88- or IL-1R-deficient mice, ample PTX3 protein may be available, but PTX3-borne signal cannot be transmitted from the surface membrane of the target cell to its nucleus where proteosynthesis controled by NF-κB takes place, including that which should supply, among other mediators, more amounts of PTX3 protein [46].

In vitro, preincubation of apoptotic cells with PTX3 enhances complement factor C1q binding and factor C3 deposition on the cellular surface, thus mimicking participation of PTX3 in complement-mediated clearance of apoptotic cells. The *in vivo* activity of PTX3, however, is dichotliomous, depending upon whether complement has been activated *systemically* in circulating blood or *locally* in the inflammatory focus. Furthermore, PTX3 binds to late apoptotic neutrophils and inhibits their phagocytosis by monocyte-derived macrophages. Consequently, $PTX3^{-/-}$ mice display increased C3 deposition in their ischemic myocardium, whereas exogenous PTX3 reduces in them complement activation, provided, of course, that the IL-1R-MyD88 pathway is intact. *In vitro,* inhibition of complement by fluid-phase PTX3 occurs at concentrations in the same range as those achieved in $PTX3^{-/-}$ mice 24 hours after myocardial reperfusion. Therefore, it can be expected that PTX3 protective activities in later phases of an AMI result from its impact on the complement cascade, which accounts for the salvage of apoptosis-destined, but still viable cells [47].

10. Pentraxin 3 Interaction with the Complement System

PTX3 is composed of a unique N-terminal region coupled to a C-terminal domain, which is homologous to the short pentraxins CRP and SAP. The N-terminal region of PTX3 is unrelated to any protein structure known so far. Studies carried out with recombinant preparations of the N- and C-terminal domains of PTX3, respectively, indicate that the N-terminal region binds ligands such as (i) fibroblast growth factor-2 (FGF2), (ii) inter-α-inhibitor, and/or (iii) conidia of *A. fumigatus*; whereas the pentraxin-like C-terminal domain of PTX3 interacts with the complement factor C1q and the adhesion molecule P-selectin. On the other hand, both

the N- and the C-terminal domains have been implicated in the interaction with the complement factor H [48].

The impact of PTX3 on complement activation is a complex issue. PTX3 binds C1q, the first component of the classical complement cascade, by interacting with the C1q globular head (gC1q) and, in particular with charged residues on the apex part of the molecule which encompass all three C1q chains (i.e., gC1qA, gC1qB, and gC1qC) [49]. PTX3 binds to *immobilized* C1q, such as is aggregated on the surface of bacterial walls. Interaction of PTX3 with surface-bound C1q leads to the *activation* of the classical pathway, as can be assessed by the deposition of C3 and C4 fragments on the target cells. PTX3 itself, however, does not interact either with C3 or with C4. In contrast, the presence of PTX3 in circulating blood leads to a dose-dependent inhibition of C1q hemolytic activity, an event in which PTX3 limits C1q binding to antibody-sensitized erythrocytes. Hence, binding of fluid-phase PTX3 to *free* C1q results in the *inhibition* of the classical cascade of complement. As it is, PTX3 exerts a dual role on the classical pathway of complement: it supports clearance of corpuscular material that binds PTX3, such as microbes or apoptotic cells, while it restricts unwanted complement activation in circulating blood [50].

The short pentraxin CRP, in addition to the classical pathway, modulates the *alternative pathway* of complement via interaction with factor H, the main soluble regulator of the alternative pathway. PTX3 can also interact with factor H, thus leading to increased factor H and factor iC3b deposition on apoptotic cells. Factor iC3b is the prevalent opsonin generated during complement activation, which performs an important activity in the process of complement-mediated phagocytosis. The net result of PTX3 impact on the alternative pathway of complement is mirrored by increased factor H deposition on PTX3-coated surfaces, that is, activation of complement by the alternative pathway in response to appropriate stimuli is increased. At the same time, however, exaggerated complement activation by the alternative pathway, which is active even under steady-state conditions to guarantee an immediate-early weapon in cases of sudden bacterial intrusion and which might, in a pathogen-free environment, inflict an erroneous damage to the host, is being held under control [51].

PTX3 interacts also with components of the lectin pathway. The mannose binding lectin (MBL) binds PTX3 via its collagen-like domain. MBL/PTX3 complexes recruit C1q and elicit C3 and C4 deposition on target cell surfaces, such as those of *Candida albicans*. Phagocytosis thereof is mightily enhanced. Furthermore, pentraxin 3 interacts with another fluid-phase PRM, namely, L-Ficolin (alternatively known as ficolin-2). Of note, ficolin-2 has been shown to bind conidia of *Aspergillus fumigatus.* This ficolin 2-*A.fumigatus* interaction is supported by PTX3 and conversely, PTX3-*A.fumigatus* binding is supported by ficolin-2. PTX3 also promotes ficolin-2-induced complement deposition on the surface of *A. fumigatus*, thus aiding the host to combat the infection [52]. Recently, an interaction between M-ficolin (ficolin-1) and PTX3 has been described in which immobilized PTX3 triggers M-ficolin- induced activation of the lectin complement pathway [53].

11. Pentraxin 3 Interaction with Microbial Moieties

Opsonization of target cells (invading pathogens or altered self structures) results in (i) facilitated pathogen recognition (and increased phagocytosis and killing) and in (ii) *innate* immune cell activation (and increased inflammatory cytokine and nitric oxide production). Moreover, opsonization by PTX3 is evidently linked to the activation of an appropriate *adaptive* immune response (i.e., dendritic cell maturation and polarization). PTX3 opsonizes manifold pathogens or pathogen-derived ligands, most importantly zymosan, *Paracoccidioides brasiliensis* or conidia of *Aspergillus fumigatus* [54]. Other interactions concern Gram-positive and Gram-negative bacteria, namely, *Staphyloccocus aureus*, *Pseudomonas aeruginosa*, *Salmonella typhimurium, Streptococcus pneumoniae,* and *Neisseria meningitidis.* Even viruses are affected, namely, human and murine cytomegalovirus (CMV) or H3N2 influenza virus.

PTX3 binds the outer membrane protein A from *Klebsiella pneumoniae* (KpOmpA). OmpA, an archetypical pathogen-associated molecular pattern, is a prominent membrane component of Gram-negative bacteria, which has been conserved in phylogeny among the *enterobacteriaceae* family. As such, KpOmpA is recognized by monocytes and dendritic cells (DCs) via two members of the scavenger receptor family (the *lectin-like oxidized low-density lipoprotein receptor-1* or LOX-1 and the *scavenger receptor expressed by endothelial cell-I* or SREC-I) and is able to induce a TLR2-dependent proinflammatory response [55]. This inflammatory response includes supplemental PTX3 production via a positive feedback loop which, in its turn, promotes the original inflammatory response brought about by KpOmpA, in terms of inflammatory cell recruitment and inflammatory cytokine release. Due to PTX3 participation, this response is complement modified [56]. In murine *Klebsiella pneumoniae* infection, the difference between death and recovery relies on PTX3-evoked fine-tuning of the balance between tumor necrosis factor (TNF-α) and nitric oxide (NO) production. By (i) shifting the balance of these antagonistic mediators in one or another way and (ii) depending upon the inaugural inflammatory response which must deal either with a *high* or a *low* bacterial inoculum, PTX3 will either favor or disfavor the ability of the host to combat the infection [57]. Essentially, these principles hold true for any inflammation, be it of microbial or sterile origin. Examples thereof will be presented followingly.

12. Pentraxin 3 and Immune Responses in *Aspergillus Fumigatus* Infection

Aspergillus fumigatus is an ubiquitary fungus which elicits a broad spectrum of diseases in immunocompromised or atopic subjects, ranging from severe pulmonary infections to allergy. Aspergillosis is a major life-threatening infection in patients with *defective phagocytosis*, the hallmark of chemotherapy- or radiotherapy-induced neutropenia and monocytopenia [58]. From among inborn immunodeficiencies,

chronic granulomatous disease (CGD) is of interest in this context. Pathophysiologically, this condition displays defective superoxide production by the enzyme NADPH. The clinical course is characterized by increased susceptibility to infections. Opportunistic pathogens such as *Aspergillus fumigatus* are involved. Prophylaxis with trimethoprim-sulfamethoxazole, itraconazole, and, in selected cases, administration of IFN-γ may be efficient, but often imperfect. Excessive inflammation is treated with systemic corticosteroids which, however, further suppress the patient's immunity. Gene-replacement therapy for patients lacking a suitable stem cell donor is still an experimental procedure. Neutrophils, although reduced in numbers and/or in function, are of paramount importance in the institution of a protective inflammatory response. Inflammation by itself may confer some protection. Notwithstanding, tight control of defense reactions is a vital condition. Unleashed inflammation is deleterious. On top of these problems, CGD patients may develop autoimmune diseases, a condition which mirrors generation of neoantigenic determinants, presumably by reactive oxygen and nitrogen species, which is the more so astonishing that the NADPH enzyme is functionally deficient [59].

In mice, genetic loss-of-function studies have yielded results that might be, at least in part, applied to humans. First, PTX3-knockout mice (*ptx3*$^{-/-}$), in whom the gene encoding for PTX3 has been silenced, are highly susceptible to invasive pulmonary aspergillosis. The course of the disease recapitulates defective recognition and killing of *aspergillus* conidia by PTX3-deficient neutrophils, dendritic cells, and alveolar macrophages [60]. Second, the p47phox$^{-/-}$ mice closely mimic human CGD, most importantly the defective, albeit detrimental inflammation. Once these mice have been infected with *Aspergillus fumigatus,* their own production of PTX3 is both delayed and insufficient. Administration of exogenous PTX3 in early infection is able to rescue antifungal resistance and, at the same time, to restrain the exaggerated inflammatory response to the fungus by way of curbing the IL-23/Th17 inflammatory axis [61]. This beneficial reversal is produced via downregulation of IL-23 production by dendritic cells and epithelial cells, an effect associated with *limited* expansion of IL-23R^{+} $\gamma\delta^{+}$ T cells. These cells are important producers of IL-17A, a mighty inflammatory cytokine and a neutrophil chemoattractant which is involved in many inflammatory diseases, once again either microbial (e.g., *Borrellia burgdorferi, Mycobacterium tuberculosis*) or sterile (atherosclerosis including its complications) in origin. Consequently to IL-17A decrease, protective Th1/T_{reg} responses are upregulated [62].

To elucidate this positive point of return, it should be reminded that defective tryptophan catabolism contributes to excessive inflammation in CGD individuals infected with *Aspergillus fumigatus.* In immunocompetent hosts, tryptophan metabolism regulates both antimicrobial resistance and tolerance to inflammation-inducing microbial stimuli. Since superoxide is a cofactor of IDO, which is the rate-limiting enzyme in tryptophan degradation, superoxide-induced activation of IDO is a central mechanism by which an equilibrium between antifungal resistance and immune tolerance is achieved. The enzyme IDO represents both the host's effector defense mechanism and a means of properly balancing the generation of *inflammatory* Th17 and *regulatory* T cells (T_{regs}) [63]. T_{regs} and IL-17-producing Th17 cells call forth opposing responses in inflammation, including aspergillosis. Whenever harmful inflammation predominates, whether microbial or sterile in origin, this IL-17-driven inflammation is associated with a decrease in anti-inflammatory T_{reg} cellular responses [64]. Basically, the same holds true for invasive aspergillosis as well as for human ischemia-reperfusion injury, no matter which organ is affected [65]. In all these cases, an attempt at a defense reaction translates into a control-evading detrimental inflammation. In terms of cellular adaptive immunity, expression of mRNA for transcription factors Tbet/Foxp3, which is indicative of Th1/T_{reg} activation, is increased in CD4^{+} T cells from wild-type, *disease-resistant* mice. Conversely, RORγt/Gata3 transcription factor mRNA expression, which is indicative of the opposite Th17/Th2 activation, is increased in CD4^{+} T cells from *disease-prone* p47phox$^{-/-}$ mice. Early administration of PTX3 to p47phox$^{-/-}$ mice restores this malevolent balance in that it reverses the original ratio of the transcription factors: it *increases* the expression of Tbet/Foxp3 and *decreases* that of RORγt/Gata3 [66]. It is tempting to speculate that comparable effects of PTX3 would be operative in patients with myocardial infarction, but reliable proofs thereof are as yet lacking.

13. Acquired Cellular Immunity in Inflammation

It is well established that CD4^{+}T cells take part in the pathogenesis of atherosclerosis [67]. In addition to classical T_h1 and T_h2 helper cellular subsets, each producing its own set of cytokines which are mutually antagonistic, a third subset has been described recently, namely, the Th17 cells [68, 69]. These cells express proinflammatory cytokines of their own. The most important of them has been named interleukin- (IL-)17. Actually, IL-17 is a common denomination for a set of closely related cytokines referred to as IL-17A to IL-17F, respectively. From the clinical point of view, IL-17A and IL-17E appear to be the most active ones [70]. The orphan nuclear receptor *retinoic acid-related orphan receptor* γt (RORγt) has been proposed as the key transcription factor for the differentiation of the Th17 lineage [71]. Th17 cells have been shown to play a role in chronic inflammation which, historically, was attributed to Th1 cells. One of these conditions is atherosclerosis [72]. Recent studies found that Th17 cells and their effector cytokine IL-17 are really increased in peripheral blood of patients with atherosclerosis, including acute coronary syndromes [73, 74]. Pathophysiologically, the Th17/IL-17^{+} cells directly support the development of atherosclerosis in ApoE$^{-/-}$ mice. Herein, the amount of Th17 cells and of RORγt in affected vessels is proportional to the magnitude of the overall atherosclerotic burden. Both mediators are not manufactured *in situ,* as can be inferred by their respective

behavior in ApoE$^{-/-}$ mice. The number of Th17 cells in spleens of young, not yet atherosclerotic ApoE$^{-/-}$ mice, has been proved comparable to that in healthy control animals. In ApoE$^{-/-}$ mice, the number of Th17 cells tended to increase as a function of time, tightly paralleling continuing development of atherosclerosis. Although CD4$^+$ T cells are the main source of IL-17, they are not the only origin of this cytokine. Neutrophils, as well as the CD8$^+$ and $\gamma\delta^+$ T cells, were demonstrated to generate IL-17 in response to the inflammatory cytokine IL-15, just to give an example. Differentiation of mature Th17 cells depends on the *stimulation* with a "cocktail" of cytokines, namely, IL-6, TGF-β, and IL-21 and on the *induction* of their inherent transcription factor RORγt. On the other hand, IL-23 is indispensable to the achievement of mature Th17 phenotype and its stability under inflammatory conditions [75, 76].

The Th17/T$_{reg}$ equilibrium is vital to the control of inflammation [77, 78]. Its derangement will bring about destabilization of atherosclerotic plaques, the composition of which has been extended recently to include Th17 cells, the cytokine IL-17A, and the transcription factor RORγt. If the fragile Th17/T$_{reg}$ balance is lost, its new inadvertent ratio will call forth the onset of acute coronary syndromes (instable angia pectoris or acute myocardial infarction). In this context, patients experience significant increase in peripheral blood Th17 cell numbers, Th17-related cytokines (IL-17A, IL-6, IL-23) and RORγt levels. At the same time, there is a decrease in blood levels of T$_{reg}$ numbers and T$_{reg}$-related cytokines IL-10 and TGFβ as well as of their transcription factor FoxP3. It may be soundly hypothesized that PTX3, which is endowed with the capacity to reverse this harmful inflammatory dysbalance in *Aspergillus fumigatus* infection by skewing the predominance of transcription factors (RORγt/Gata3 → Tbet/Foxp3) with consequent shift of their respective cellular subsets (Th17/Th2 → Th1/T$_{reg}$), might bring about a comparable beneficial effects in this form of sterile inflammation. Nevertheless, it must be strongly pointed out that results yielded by experimental animals cannot by dogmatically extrapolated to humans, as attractive as they may appear [79].

14. Concluding Remarks

In both acute infections and acute coronary syndromes, the role of PTX3 appears to be primarily protective in that the long pentraxin dampens the inappropriate, exaggerated inflammatory response. On the local level, PTX3 released by PMNs or produced by monocytes/macrophages will abolish neutrophil recruitment and oxidative burst, a condition which kills bacteria by reactive oxygen species, while it inflicts collateral damage to the host. PTX3 co-operation with the adaptive immune system in defense inflammation which combats infection supports, in its turn, development of a protective Th1/T$_{reg}$ immune response while at the same time it limits harmful inflammation elicited by the Th17/Th2 immune response. Further studies are needed in this context to furnish data that would imply to which extent PTX3 might be employed therapeutically.

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Review Article

Diverse Roles of Macrophages in Atherosclerosis: From Inflammatory Biology to Biomarker Discovery

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Cardiovascular disease, a leading cause of mortality in developed countries, is mainly caused by atherosclerosis, a chronic inflammatory disease. Macrophages, which differentiate from monocytes that are recruited from the blood, account for the majority of leukocytes in atherosclerotic plaques. Apoptosis and the suppressed clearance of apoptotic macrophages (efferocytosis) are associated with vulnerable plaques that are prone to rupture, leading to thrombosis. Based on the central functions of macrophages in atherogenesis, cytokines, chemokines, enzymes, or microRNAs related to or produced by macrophages have become important clinical prognostic or diagnostic biomarkers. This paper discusses the impact of monocyte-derived macrophages in early atherogenesis and advanced disease. The role and possible future development of macrophage inflammatory biomarkers are also described.

1. Introduction

Cardiovascular disease (CVD) is the leading cause of mortality in developed countries and is likely to attain this status worldwide, accounting for 16.7 million deaths each year [1, 2]. Coronary artery disease (CAD) and cerebrovascular disease are the most common forms of CVD, whose underlying pathological feature is atherosclerosis. Atherosclerosis is a slowly progressing chronic disease of large and medium-sized arteries which is characterised by the formation of atherosclerotic plaques consisting of necrotic cores, calcified regions, accumulated modified lipids, migrated smooth muscle cells (SMCs), foam cells, endothelial cells (ECs), and leukocytes [3].

Since the term arteriosclerosis was first introduced by Jean Lobstein in 1829 [4], it has long been believed that atherosclerosis involved the merely passive accumulation of cholesterol in arterial walls. In the 1970s, the response-to-injury model was described [5]. Today, the picture of atherosclerosis is much more complex as it has been considered a chronic inflammatory disease, involving both the innate and adaptive immune systems, which modulate the initiation and progression of the lesions, and potentially devastating thrombotic complications [6]. Understanding the principles of the inflammatory processes is important for deciphering the complex processes involved in atherosclerosis progression. Atherosclerotic plaques are characterised by an accumulation of lipids in arterial walls together with infiltration of immunocytes. The degree of influx of inflammatory cells to atherosclerotic lesions is determined based on monocyte recruitment, macrophage egress, and the balance of proliferation, survival, and apoptosis within the arterial walls [7].

Macrophages are the first inflammatory cells to invade atherosclerotic lesions, and they are the main component of atherosclerotic plaques [8]. Inflammatory cytokines produced by macrophages stimulate the generation of endothelial adhesion molecules, proteases, and other mediators, which may enter systemic circulation in soluble forms [9]. Cytokines as inflammatory biomarkers, independent of cholesterol and regulators of blood pressure, could yield more information on different aspects of pathogenesis of atherosclerosis [10]. This paper discusses the central roles of macrophages in every stage of atherosclerosis, focusing on

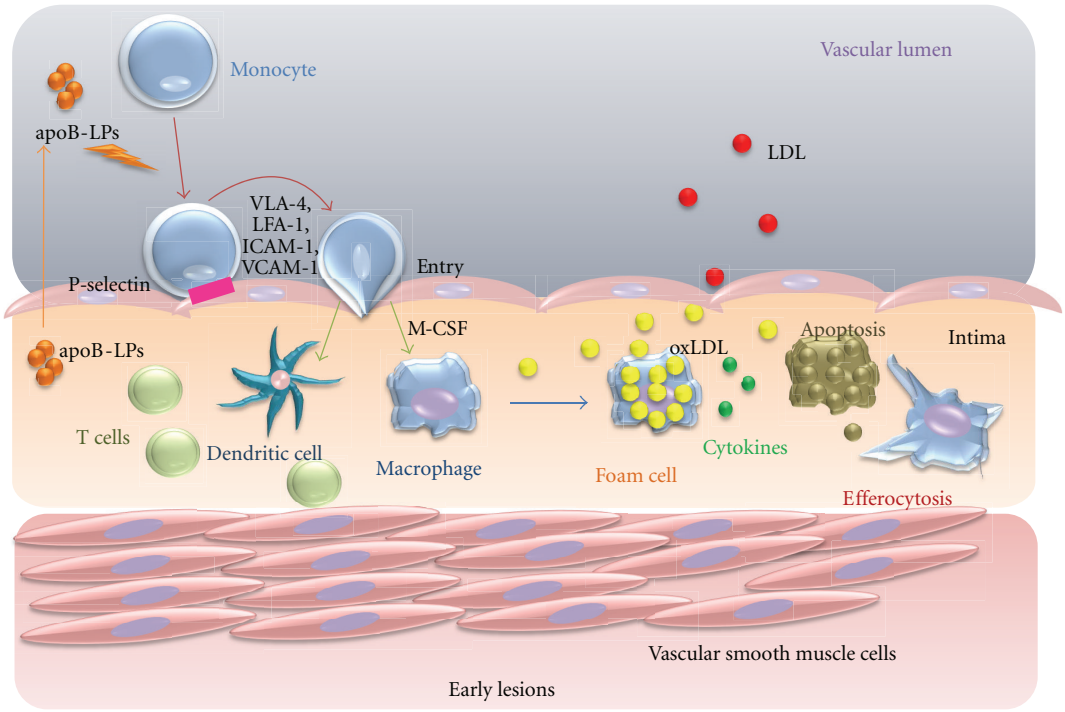

Figure 1: The roles of M1 and M2 macrophages. Ly6C high monocytes differentiate into M1 type, classically activated macrophages that affect proteolysis and produce antibacterial products. Ly6C low monocytes differentiate into M2 type, alternatively activated macrophages that are involved in wound repair and tissue remodelling. M1 and M2 cells secrete different cytokines that function in efferocytosis and the formation of foam cells.

the role of inflammatory biomarkers in predicting primary cardiovascular events related to macrophages.

2. Initiation and Early Progression of Atherosclerosis

2.1. Recruitment and Entry of Monocytes to Arterial Walls. Monocytes originate from bone marrow-derived progenitor cells and do not proliferate in the blood [11]; their functions under homeostatic conditions remain unclear. The mechanisms of monocyte homing to healthy aortas are not well defined; more is known about monocyte recruitment into aortas during atherogenesis [12]. During the pathogenesis of atherosclerosis, blood monocytes infiltrate from blood to the intima and subintima [13], a process which is activated by subendothelial accumulation of apolipoprotein B-containing lipoproteins (apoB-LPs) [14]. Summoned by chemokinesis, monocytes roll over and become tethered to endothelial cells overlying retained apoB-LPs through interactions between monocyte P-selectin glycoprotein ligand-1 (PSGL-1) and endothelial selectins [14]. E-selectin overlaps with P-selectin to support rolling [15]. After monocytes roll on the inflamed aortic endothelium, they use lymphocyte function-associated antigen-1 (LFA-1), very late antigen-4 (VLA-4) and their respective endothelial cell ligands, including vascular cell adhesion molecule (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1), to slow rolling and form tighter adhesions [16]. Finally, firm adhesion is followed by entry of monocytes into the subendothelial space (diapedesis) [17] (Figure 1).

In mice, monocytes can be identified from other circulating cells by the differential expression of chemokine C-C motif receptors 2 (CCR2), chemokine C-X3-C motif receptor 1 (CX3CR1), and Ly6C antigen, which is monocyte/macrophage cell differentiation antigen regulated by interferon gamma [11]. Apolipoprotein E–/– (*Apoe–/–*) mice, a model system for atherosclerosis, are prone to develop atherosclerosis because they have high levels of the atherogenic lipoprotein known as remnant lipoprotein [18]. $Ly6C^{high}CCR2^{+}CX3CR1^{Low}$ monocytes, which are precursors of inflammatory macrophages, have been observed to adhere to activated endothelium in *Apoe–/–* mice [19]. In contrast, little is known about how a lack of apoE affects inflammatory $Ly6C^{low}CCR2^{-}CX3CR1^{high}$ monocytes [20]. These studies suggest that there is persistent recruitment of inflammatory monocytes into established atherosclerotic lesions (Figure 2). These studies described above are limited in mice and it may be difficult to interpret human macrophage subsets, but two major subsets of human macrophages can be defined: $CD14^{high}CD16^{low}$ macrophages typically represent 85% ~ 95% monocytes in healthy individuals; $CD14^{low}CD16^{high}$ macrophages are comprised in the remains [21]. The role of each subset in human atherosclerosis remains unknown.

2.2. Monocyte Differentiation into M1 and M2 Subsets of Macrophages. Driven by macrophage colony-stimulating

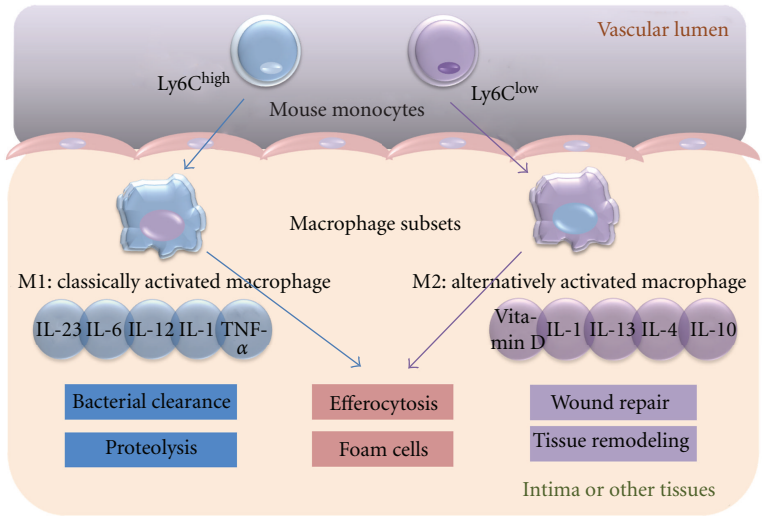

Figure 2: Signalling pathways in a macrophage involved in atherosclerosis. Pro- and anti-inflammatory factors act on macrophages, leading to activation of downstream scavenger receptors (SRs)/toll-like receptors (TLRs)-NF-κB signalling, endoplasmic reticulum (ER) stress and efflux of cholesterol via ABCA and ABCG transporters.

factor (M-CSF) and other differentiation factors, monocytes differentiate into two major types of macrophages and/or dendritic cells [22, 23]. M1 and M2 macrophages play opposite roles during inflammation, although both are present in atherosclerotic lesions. M1 macrophages, which are differentiated from Ly6C^{high} monocytes and promote inflammation, are classically activated by lipopolysaccharide in the presence of IFN-γ, leading to the production of high levels of IL-2, IL-23, IL-6, IL-1, and TNF-α. In contrast, activated M2 macrophages, which are differentiated from Ly6C^{low} monocytes and promote resolution inflammation, differentiate in the presence of IL-4, IL-13, IL-1, or vitamin D3 and tend to produce a large amount of IL-10 and express scavenger receptors, mannose receptors, and arginase [24] (Figure 2). Recently, there has been a great deal of interest in macrophage heterogeneity in atherosclerotic lesions, particularly regarding the roles of M1 versus M2 macrophages. There is evidence that an imbalance in the ratio of classically activated M1 and alternatively activated M2 macrophages in advanced atherosclerosis impair resolution *in vitro* [25], but a clear picture has not yet emerged from these studies [23]. Most of the hypotheses in this area have been driven by *in vitro* studies exploring gene expression patterns and functional attributes of monocytes or macrophages subjected to various treatments, including growth factors, cytokines derived from helper T cells [26], the transcription factors peroxisome proliferators-activated receptors (PPARs) γ [27], and the bioactive lipid sphingosine-1-phosphate [28]. However, there is a significant difference between *in vitro* and *in vivo* results, which makes atherogenesis more complex. Future projects should focus on the characterisation of macrophage heterogeneity with respect to differential expression of specific molecular biomarkers that have functional significance for atherogenesis [29]. Additional attention should be paid to the roles of cytokines in controlling monocytes that differentiate into dendritic cells (DCs) rather than macrophages.

2.3. Important Receptors and Transporters for Cholesterol Loading and Efflux in the Toll-Like Receptors of Macrophages. In the innate immune system, toll-like receptors (TLRs) are the primary receptors that recognise highly conserved structural motifs of pathogens [30]. Under hyperlipidemic conditions, TLRs likely participate in the regulation of atherosclerosis. The activation of TLRs induces the production of proinflammatory cytokines and nitric oxide in macrophages and the induction of DC maturation, leading to the upregulation of costimulatory molecules, such as CD80 and CD86. In addition, TLR1, TLR2, TLR4, and TLR6 are expressed in atherosclerotic lesions. A large number of pathogen-associated molecules can activate TLRs. Heat-shock proteins (hsp60) [31] and oxidised (ox) LDL [32] mediate at least a part of their effects within atherosclerotic plaques through TLR4 binding. TLR2, expressed on cells that do not derive from bone marrow, appears to promote atherogenesis in mice [33]. Interestingly, Sun et al. showed that free cholesterol (FC) accumulation in the endosomal compartment increases the inflammatory response in a TLR-dependent fashion, and TLR3 is the predominant receptor involved in this process [34] (Figure 3).

2.4. Scavenger Receptors. Macrophage scavenger receptors (SRs) are found to bind and internalise modified forms of LDL through mechanisms that are not inhibited by cellular cholesterol content, and they are likely responsible

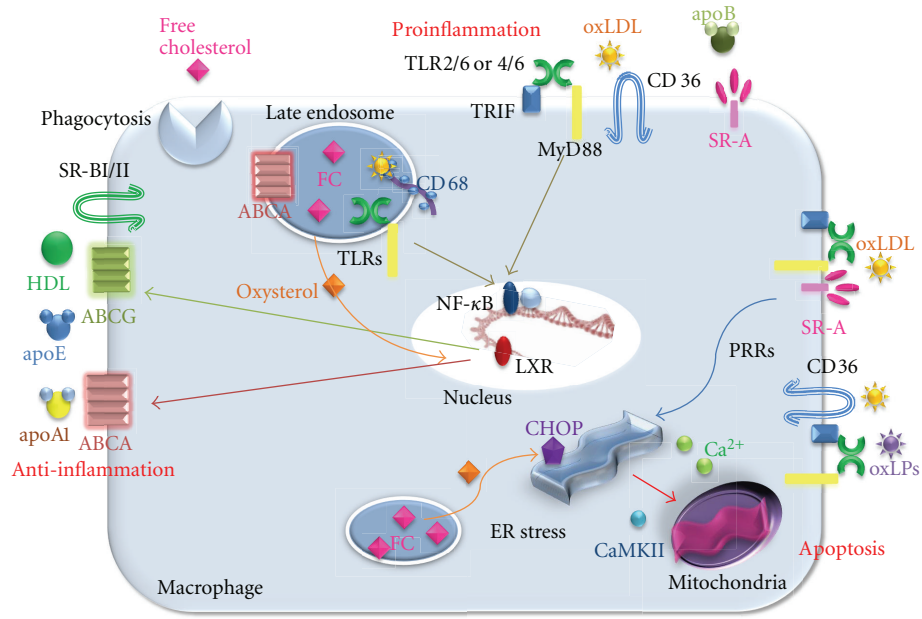

Figure 3: The fate of macrophages in an early lesion of atherosclerosis. The accumulation of apolipoprotein B-lipoproteins (apoB-LPs) in the matrix beneath the endothelial cell layer leads to the recruitment of monocytes. The cells differentiating into macrophages undergo foam cell formation, leading to apoptosis. Because efferocytosis works efficiently, this lesion does not develop necrotic core. The resolution of the inflammation results in decreased plaque progression.

for macrophage cholesterol accumulation [35]. SR class A (SR-AI and AII), expressed on the surface of macrophages, account for the uptake of acetylated LDL in the majority of macrophages, but macrophages preferentially bind oxLDL, recognising the modified apoB components of the particles [36]. Interestingly, SR-As expression is increased in animals with low atherosclerotic responses, suggesting that this pathway is protective. Furthermore, overexpressing a secreted form of the extracellular domain of human SR-A resulted in a 20% reduction in monocyte/macrophage adherence to endothelial cells in atherosclerotic lesions in *Ldlr*–/– mice [37]. Thus, the use of such decoy SRs may prove beneficial for retarding the development of early atherosclerotic lesions (Figure 3).

Other studies indicate that SR class B CD36 plays a major role in the clearance of oxLDL, contributing 60% to 70% of cholesterol ester accumulation in macrophages exposed to LDL oxidised by Cu^{+2} and myeloperoxidase/peroxynitrite [38, 39]. CD36 activates signalling via TLR2 and TLR6 in response to lipoteichoic acid and diacylated macrophage-activity lipopeptide 2 [40, 41]. In addition, a newly described TLR heterodimer of TLR4/6 has been shown to cooperate with CD36 in activating NF-κB in response to oxLDL [42] (Figure 3).

SR-BI and SR-BII share 30% sequence homology with CD36 and both can bind modified forms of LDL as well as native HDL, LDL, and VLDL [43] (Figure 3). These receptors have a major impact on lipoprotein metabolism through two mechanisms: (1) SR-BI mediates cholesterol transfer to HDL, and (2) SR-BI facilitates selective delivery of lipoproteins from HDL to steroidogenic tissues for excretion into bile and feces in the liver [35]. Although the antiatherogenic effects of SR-BI have been largely attributed to mediation of cholesterol ester uptake in the liver, this receptor is highly expressed on foam cells in human and mouse atherosclerotic lesions, where it may influence lesion development by affecting both the uptake of lipoproteins and the efflux of cholesterol to HDL [44]. The other class D SRs, CD68, and its murine homolog macrosialin are predominantly expressed in late endosomes and lysosomes of macrophages and may play a role in endolysosomal processing for oxLDL [45] (Figure 3).

2.5. ATP-Binding Cassette Transporters, Subset A and G (ABCA and ABCG). Free cholesterol released from lysosomes and rehydrolysed cholesteryl ester droplets can also traffic to the plasma membrane and thus be available for efflux out of the cells [46]. Cholesterol efflux is thought to be a major process involved in plaque regression when hypercholesterolemia is reversed. The mechanisms and exact route of cholesterol transport to the plasma membrane are not fully known, although Golgi-to-plasma membrane vesicular transport may be involved [47]. Once at the plasma membrane, cholesterol is transferred to the outer leaflet, where it is removed from cells by ABCA1- and ABCG1-mediated transport to apolipoprotein A1 and HDL, respectively, or by "passive diffusion" to cholesterol-poor HDL [48]. As predicted, genetic deficiencies of ABCA or ABCG could account for enhanced inflammation in atherosclerosis, especially after treatment with TLR ligands [49] and result in foam cell formation and further acceleration of atherosclerosis [50].

Extensive work *in vitro* and *in vivo* has focused on how sterol-regulated transcription factors, liver X receptors LXRa and LXRb (LXR), induce ABCA1 and ABCG1 and promote regression of foam cell lesions through this and other mechanisms [51]. Free cholesterol (FC) within macrophages has recently been proposed as an initiator of a proinflammatory signalling response in developing atherosclerotic lesions [52]. Oxysterols, from FC phagocytosis, are LXR agonists and increase reverse cholesterol transport (RCT) from macrophages by increasing expression of macrophage apolipoprotein E (apo E) and the cholesterol efflux transporters ABCA1 and ABCG1. This is likely an important part of the mechanism for LXR-dependent protection from atherosclerosis because these effects are not observed in LXR knockout mice [53]. Because accumulation of FC within macrophages at sites of atherosclerotic lesions converts them into foam cells [54] by stimulating RCT, LXR reduces foam cell formation and lesion cholesterol content directly (Figure 3). As a therapeutic strategy to promote lesion regression, investigators have attempted to enhance macrophage cholesterol efflux by increasing HDL or HDL-like particles or by increasing ABC transporters [48]. Though no drugs have yet been approved for this purpose, this approach continues to be a major focus of cardiovascular drug discovery.

2.6. Apoptosis of Macrophages in Early Atherosclerotic Lesion. The mechanism and role of macrophage apoptosis in early lesions are still not well understood. It is difficult to detect macrophage apoptosis in early lesions because apoptotic cells are rapidly cleared by the adjacent macrophages through phagocytosis (known as efferocytosis), which will be described later in the section of advanced progression in atherosclerosis (Figure 1). Several studies determined the effect of apoptosis on the progression of atherosclerosis. *Ldlr*–/– mice develop high levels of LDL when placed on a high-fat diet, because their hepatocytes lack LDL receptors and thus cannot efficiently eliminate the atherogenic LDL particles from the blood [55]. In *Ldlr*–/– mice in which bone marrow derived cells, including regional macrophages, are deficient of the proapoptotic protein Bax, the aortic lesions showed decreased macrophages apoptosis. Additionally, these lesions were larger and more macrophage-rich [56]. Conversely, *Ldlr*−/− mice, which lack the prosurvival protein AIM, showed an increase in apoptosis of early regional macrophages and developed smaller atherosclerotic lesions [57]. Thus, the apoptosis of the early regional macrophages is associated with lesion size and plaque progression. Deficiency of phospholipase Cβ3 resulted in enhanced sensitivity of newly recruited macrophages to oxLDL-induced apoptosis in early lesions, accompanied by a concomitant decrease in atherosclerosis [58]. Because knocking out phospholipase Cβ3 does not appear to change the mouse phenotype, this may be an attractive target to modulate macrophage apoptosis.

3. Advanced Progression in Atherosclerosis

Macrophages in advanced atherosclerosis contribute to the plaque morphology, thinning the fibrous cap, and necrotic core, which can lead to increased pro-inflammatory responses and further apoptotic signals for SMCs, ECs, and leukocytes within the plaques [59]. The vulnerable plaque is prone to rupture and induction of thrombosis. In autopsy specimens containing atherosclerotic lesions, rupture sites were responsible for the acute vascular events [60]. The rupture sites, which are located on the shoulder of raised lesions, are almost always in the areas close to plaques' necrotic cores, and are associated with the thinning of fibrous caps. One of the most important questions in atherosclerosis is how macrophages contribute to this advancement in plaque progression (Figure 4).

Macrophages decrease intimal myofibroblast-like SMCs and degradation of collagens [61] (Figure 4). *In vitro* data show that macrophages can trigger apoptosis of SMCs by activating the Fas apoptotic pathway and secreting proapoptotic TNFα and nitric oxide [62]. Macrophages may also decrease collagen synthesis in intimal SMCs through the secretion of macrophage-derived matrix metalloproteinases (MMPs) to decrease collagen synthesis [63]. MMPs may also be involved in thinning of the fibrous cap. In a study that attempted to look directly at plaque disruption, macrophage overexpression of MMP-9 had little effect on *Apoe*−/− mice due to a lack of MMP activation in plaques, but the overexpression of a constitutively active mutant form of MMP-9 resulted in plaque fissures [64]. Further details about TNFα and MMPs are discussed below in the biomarkers section.

3.1. Plaque Necrosis and Macrophage Death in Advanced Atherosclerotic Lesions. Plaque necrosis contributes to inflammation, thrombosis, plaque breakdown, and physical stress on the fibrous cap [65]. Necrotic cores arise from the combination of apoptosis of macrophages and the phagocytic clearance of the apoptotic cells in advanced plaques [18]. There is emerging evidence that SR-A plays different roles in early and advanced atherosclerotic lesions. As we described previously, SR-A has the protective function in early lesions. However, in advanced atherosclerotic lesions, in which macrophage cell death leads to necrotic core formation and plaque destabilisation, SR-A may have important roles in both the induction of apoptosis and clearance of these dying cells. In hypercholesterolemia, macrophage pathways for metabolising modified lipoproteins are thought to be overwhelmed, leading to a toxic accumulation of free cholesterol in the cells that result in the endoplasmic reticular stress. In this setting, the engagement of SR-A pathways by modified lipoproteins or fucoidan triggers apoptotic cell death, indicating that the SR-A signalling contributes to macrophage death and necrotic core formation [66]. However, this proatherosclerotic role is also balanced by the ability of SR-A to recognise and clear apoptotic cells in a nonphlogistic manner. These additional functions of SR-A must be considered when proposing therapies to inhibit this pathway. Longer-term studies of SR-A manipulation will be required to determine the impact of this receptor at later stages of atherosclerosis.

A number of processes in advanced lesions may trigger macrophage death, and it is almost certain that a combi-

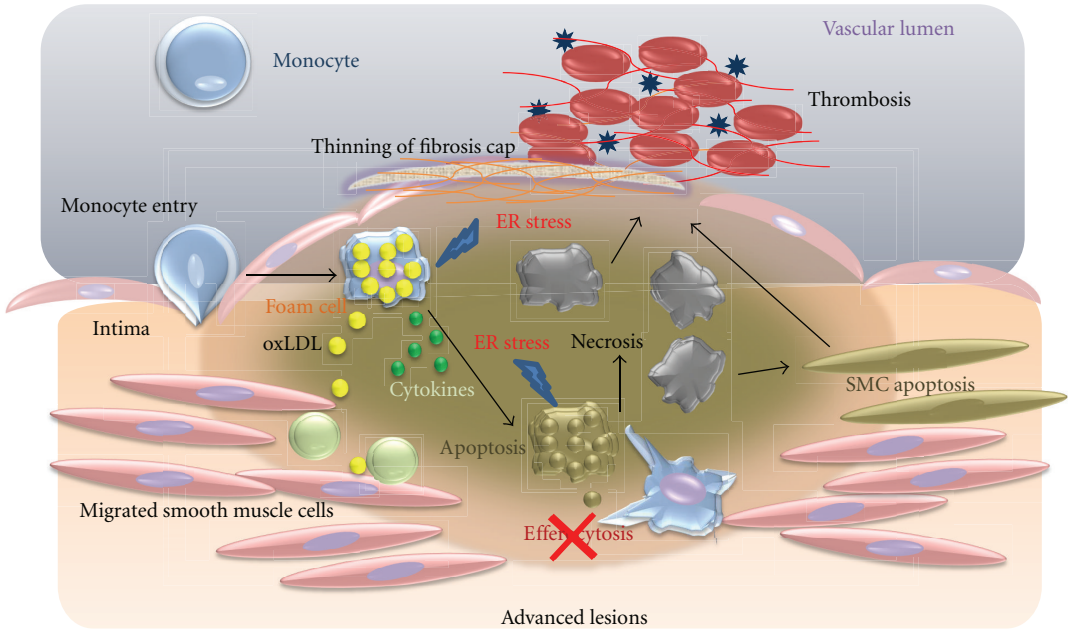

Figure 4: Cellular interactions with macrophages in an advanced lesion. Foam cells accumulate in the intima and undergo apoptosis that is triggered by cytokines. Efferocytes do not function properly, and apoptotic cells secondarily become necrotic cells, contributing to the formation of a necrotic core. Necrosis of macrophages and SMCs decrease collagen synthesis to diminish the collagen content of the fibrous cap, triggering rupture and thrombosis.

nation of factors and processes plays a role *in vivo*. A potential role for these processes is supported primarily by studies with cultured macrophages. The endoplasmic reticulum (ER) stress, primarily established by Tabas laboratory, may lead significantly to macrophage apoptosis and generation of necrotic core [67]. The high levels of ER stresses, such as intracellular oxysterols, lead to activate the unfolded protein response (UPR) pathway, which increases the expression of a proapoptotic protein, called CEBP-homologous protein (CHOP) [68]. The elevation of CHOP can trigger macrophage apoptosis by several mechanisms, but recent work shows a specific apoptotic mechanism involving calcium channel activity in the ER lumen [69]. Most importantly, a deficiency of CHOP in the models of advanced atherosclerosis suppresses advanced lesions due to macrophage apoptosis and plaque necrosis. Calcium released from the ER can trigger apoptosis through excess uptake into mitochondria that activates calcium/calmodulin-dependent protein kinase II (CaMKII), which, in turn, promotes cell apoptosis by activating both Fas death receptor and mitochondrial membrane permeabilization [69]. Another system may provide subtle ER stress, in which a "second hit" is needed to trigger apoptosis [70] (Figure 3). In this system, ER stress and macrophage apoptosis are induced by low-dose ER stressors including thapsigargin or 7-ketocholesterol, and combination of pattern recognition receptors activation as the "second hits", each of which is unable to induce apoptosis by themselves [70] (Figure 3). An example of PRR activation is activators of SR-A and TLR 4, such as oxLDL. The other experiment demonstrated that activators of CD36 and TLR2/6, such as oxLDL and oxidized PLs (oxPL), can enhance the apoptosis pathways [67] (Figure 3). The role of SR-A and CD36 as the "second hits" for ER stress-induced apoptosis was demonstrated by a mouse model in which these receptors were targeted, with a result that apoptosis of advanced regional macrophages and plaque necrosis were deceased [71]. In humans, advanced plaques show similar results to those seen in mice. Autopsy specimens from human coronary arteries with heart disease showed a correlation with expression of markers of the UPR, including CHOP, apoptosis, and advanced plaque stage [72].

Notably, macrophage apoptosis does not trigger plaque necrosis. Plaque necrosis and rupture occurs when apoptotic cells are not cleared sufficiently. Tabas called this phenomenon efferocytosis, which describes the phagocytic clearance of apoptotic cells [18]. Efferocytosis in early lesions prevents cellular necrosis and triggers anti-inflammatory pathways through TGF-β and the activation of the NF-κB cell survival pathway (Figure 1) [73]. However, how efferocytosis becomes defective in advanced lesions is still unknown. It is assumed that the efferocytosis does not occur in advanced lesions, resulting in defective anti-inflammatory signalling (Figure 4) [18].

4. Biomarkers as Risk Factors Associated with Macrophages in Atherosclerosis

Given the new understanding of inflammation in atherosclerosis and their central role of macrophages, inflammatory biomarkers for disease progression in atherosclerosis should be independent of cholesterol and regulators of blood. In this regard, we will discuss biomarkers related to

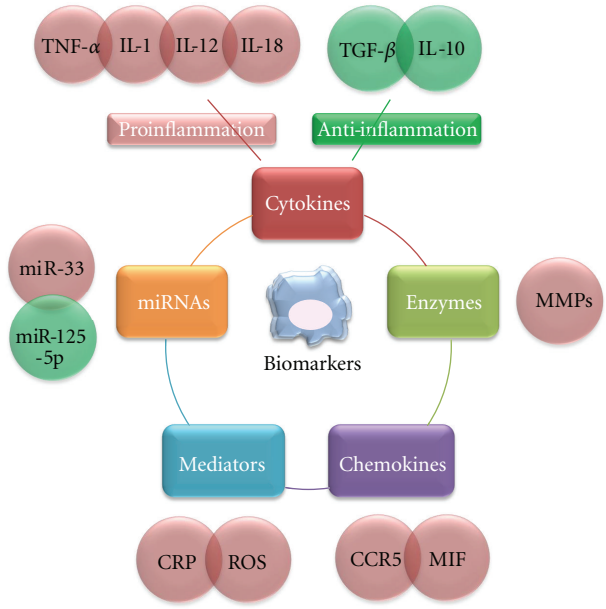

Figure 5: Pro- and anti-inflammatory biomarkers and microRNAs related to macrophages. All of these inflammatory markers and mediators, released at different stages of progression in atherosclerosis, can enter the circulation, affecting the prognosis of patients with atherosclerosis.

macrophages and inflammation in atherosclerosis (Figure 5). As our understanding of the biology of atherothrombosis has improved [74], several studies have evaluated a series of candidate biomarkers of inflammation, oxidative stress, and thrombosis as potential clinical tools to improve the prediction of risk in atherosclerosis [75, 76]. Although there are hundreds of papers that discuss the important functions of many mediators of atherosclerosis, the distinction between biomarkers versus mediators of disease has proven quite confusing. As discussed above, a particular molecule may participate clearly in a pathogenic pathway but not serve as an effective biomarker. For example, soluble VCAM-1 is not a useful indicator of risk of future myocardial infarction in apparently healthy men [77]. However, researchers have demonstrated that VCAM-1 is essential for the initiation of an atherosclerotic lesion [78].

4.1. Involvement of Cytokines Secreted by Macrophages in Atherosclerosis

4.1.1. Tumour Necrosis Factor-α (TNF-α). TNF-α regulates a number of critical cell functions including cell proliferation, survival, differentiation, and apoptosis. Macrophages produce TNF-α induced by TLRs and are also highly responsive to TNF-α through the TNF receptor (TNFR) [79, 80]. One of the various functions is a pivotal role in orchestrating the production of a pro-inflammatory cytokine cascade. TNF-α is thus considered to be a "master regulator" of pro-inflammatory cytokine production [81]. TNF-α-deficient *Apoe*−/− mice show a reduction in lesion formation, with a concomitant decrease in VCAM-1 and ICAM-1 expression, which are important for monocyte rolling on endothelial cells as mentioned previously [82]. In contrast, mice deficient in the TNF-α receptor (TNFR) develop larger lesions than control mice [83]. In addition to these roles, Witsell and Schook [84] demonstrated that TNF-α has macrophage differentiation capabilities. TNF-α gene transcripts are expressed during differentiation of bone-marrow-derived macrophages. TNF-α affects the development of atherosclerosis at the fatty streak stage, and cleavage of TNF is an important step in activating the proatherogenic properties of TNF-α [85].

4.1.2. Interleukin 1 (IL-1). IL-1 stimulation initiates leukocyte adhesion to ECs for macrophage transmigration and contributes to slowly progressing inflammatory processes that take place in atherosclerosis [86]. Studies involving blocking IL-1ra antibodies in *Apoe*−/− mice and with *Ldlr*−/− transgenic mice that overexpress IL-1 or that have a deficiency in IL-1β clearly show that IL-1 is involved in atherogenesis [87]. Yet, although the circulating levels of IL-1, even in severe inflammatory diseases, are undetectable, the availability of anti-IL-1 antibodies will likely be very useful in the future [86].

4.1.3. IL-12. IL-12 is a key Th1 cytokine that is produced mainly by plaque macrophages and stimulates the proliferation and differentiation of NK cells and T cells. IL-12 is detected in the aortas of *Apoe*–/– mice, and the administration of IL-12 results in enhanced lesion size in *Apoe*−/− recipients [88]. IL-12 p40-deficient *Il12b*−/−*Apoe*−/− mice have a 52% reduction of the plaque area at 30 weeks, but not at 45 weeks of age [89]. T lymphocyte recruitment into the intima was accelerated in early and advanced atherosclerotic lesions [16]. Most of the T cells are TCRαβ CD4+ cells with an activated phenotype, and a few express CD8+ or TCRγδ [90]. Interestingly, analysing CD4+ T cells showed that IL-12 upregulates CCR5 expression, chemotaxis, and transendothelial migration toward CCL5 through IL-12 receptors [91].

4.1.4. IL-18. IL-18 is produced by macrophages and administration of IL-18 antibodies accelerates development of atherosclerotic lesions in Apoe−/− mice. IL-18 seems to enhance atherosclerosis by increasing IFN-γ [92]. Although IL-18 is not currently considered a useful tool for the presence of subclinical atherosclerosis in general population [93], the AtheroGene Study indicates that high serum concentrations of IL-18 likely cause cardiovascular death in patients with coronary artery disease [94].

4.1.5. Soluble CD40 Ligand (CD40L). Macrophages, T lymphocytes, ECs, SMCs, and DCs express CD40L, whereas CD40 is found on macrophages, ECs, and SMCs from atherosclerosis-prone vessels [9]. The interaction of CD40 with CD40L plays a significant role in thrombosis, but it also contributes to modulation of the immune response in plaques. Treatment with antibodies against CD40L reduces atherosclerosis in *Ldlr*−/− mice, with a concomitant

decrease in macrophages and T cells and a reduction in VCAM-1 expression [95]. Further experiments using *Cd40lg−/−Apoe−/−* mice have demonstrated a proatherogenic role for CD40L in advanced atherosclerosis by promoting lipid core formation and plaque destabilisation [96]. Because preanalytical sampling conditions critically influence the soluble CD40L concentration, only plasma samples are appropriate for CD40L measurement [97]. Although CD40L is critical for the development of advanced lesions in animal experiments, the Dallas Heart Study suggests that CD40L is not identified in subclinical atherosclerosis in the general population [98]. However, high concentrations of CD40L are associated with increased vascular risk in healthy women according the results of the Women's Health Study [99].

4.2. Anti-Inflammation Factors

4.2.1. IL-10. IL-10, which is derived from monocytes and macrophages, is an important anti-inflammatory regulator for the development of advanced atherosclerosis. As expected, IL-10-deficient mice showed a decreased amount of collagen, induced by IFN-γ production in the atherosclerotic vessels [100]. Studies with *Il10−/−Apoe−/−* mice confirmed the atheroprotective properties of IL-10 in early stage atherosclerosis and showed that IL-10 promotes the stability of advanced plaques [101]. IL-10 is not a prognostic marker for cardiovascular diseases. Although, it is possible to test serum concentrations of IL-10, we anticipate future studies on the involvement of this marker.

4.2.2. TGF-β. Several cell types, including macrophages, produce TGF-β. Studies with animal models suggest that local (rather than systemic) alterations in TGF-β activity may be important during atherogenesis and that TGF-β levels in tissues may be more informative than those in blood [102]. *Apoe−/−* mice that express a dominant-negative form of TGF-β receptor II in T cells clearly demonstrated substantial roles for TGF-β in controlling the Th1 response in atherosclerosis [103]. Several studies suggest that TGF-β levels are reduced at sites of atherosclerotic plaque development. Introducing blocking antibodies against TGF-β or treatment with soluble TGF-β receptor II accelerates atherosclerosis with a significant loss of collagen content [87]. Although a direct measure of the ligand is technically demanding, associations between heart disease and genetic polymorphisms that are known to modulate ligand production might prove more accessible. Furthermore, such associations would support a causal relationship between altered TGF-β production and diseases [102]. A number of studies have examined the association between these polymorphisms and cardiovascular disease status. A large study of more than 6000 individuals who were involved in the Rotterdam study found an association between TGF-β1 polymorphisms and stroke (another pathology associated with plaque rupture, but in a different vascular field) [104]. Recently, using autopsy sections of atherosclerosis in a Japanese population, Oda et al. observed a significant association between atherosclerosis and the only TGF-β1 gene polymorphism, at least in some artery fields [105]. Taken together, these studies suggest that decreasing production of TGF-β1 ligands might favour unstable lesion phenotypes without affecting the plaque burden, once again highlighting the need to carefully select the cardiovascular endpoint under study.

4.3. Chemokines Produced by Macrophages in Atherosclerosis

4.3.1. Chemokine Receptor CCR5. CCR5 was initially known as a coreceptor on macrophages for HIV infection. However, evidence now supports a role for CCR5 and its ligands CCL3 (MIP-1a), CCL4 (MIP-1b), and CCL5 (RANTES) in the initiation and progression of atherosclerosis [106]. Although there is no CCR5 in normal coronary arteries, CCR5 immunoreactivity is detected in atherosclerotic lesions, suggesting colocalisation of VSMC with macrophages [107]. It has been suggested that CCR5 may be more important in the later stages of plaque development [108]. A recent study found more than 50% reduction in the size of plaque lesions in the aortic root and the abdominal aorta of *Apoe−/−Ccr5−/−* mice and fewer macrophages in lesions compared with *Apoe−/−* mice [109]. The combined inhibition of three chemokine–receptor systems, MCP-1 (CCL2)/CCR2, fractalkine (CX3CL1)/CX3CR1, and CCL5/CCR5, was reported to abolish development of atherosclerosis in an *Apoe−/−* mouse model [110], supporting nonredundancy of these chemokines with regard to monocyte mobilisation in atherosclerosis. Compared with chemokine receptors, the ligands CCL3, 4, and 5 seem to be better choices for biomarkers in atherosclerosis because it is possible to test their mRNA levels in circulating leukocytes. The role of CCL3 and CCL4 acting on CCR5 in atherogenesis is less well defined, but these chemokines also appear to be important in atheroma progression and inflammatory cell recruitment into plaques [111]. In particular, findings from animal models indicate that CCL5 plays a greater role in the development of atherosclerotic plaque than other CCR5 ligands [112].

4.4. Macrophage Migration Inhibitory Factor (MIF).

MIF is produced by macrophages in early and advanced atherosclerotic lesions. The role of MIF with respect to inflammatory cell recruitment in atherosclerotic plaque progression has been described [113]. A study of *Mif−/−Ldlr−/−* mice suggested that MIF is involved in atherosclerosis through the regulation of lipid deposition, protease expression, and intimal thickening [114]. Because MIF can be readily measured in plasma and other tissue fluids in different disease states [115], the different roles of MIF as a biomarker in pathogenesis and progression of atherosclerosis are an important area of inquiry.

4.5. Inflammation-Regulating Enzymes: Matrix Metalloproteinases (MMPs).

Macrophage-derived MMPs are involved in the thinning of the fibrous cap [116]. MMPs are a family of protease-activated enzymes that degrade extracellular matrix (ECM) proteins. The regulation of MMPs is complex; once

activated, an MMP can activate others. Studies showing a temporal and spatial correlation between the presence of macrophages in shoulder plaque regions, thinning of the fibrous cap in these regions, MMP-2 and MMP-9, have stimulated great interests in the potential roles of MMPs in plaque rupture [117]. According to the follow-up data, plasma MMP-9 during acute coronary syndromes is increased two to three times compared with controls [118]. However, whether MMP-9 becomes the independent prognostic marker still requires further and large-scale research. A targeted approach that inhibits MMPs has already been considered [119].

4.6. Proinflammatory Mediators Associated with Macrophage: C-Reactive Protein (CRP). Multiple large-scale studies demonstrate that CRP strongly and independently predicts adverse cardiovascular events, including myocardial infarction, ischemic stroke, and sudden cardiac death because of atherosclerosis [120, 121]. However, these mechanisms have not been comprehensively identified. CRP is found close to LDL and macrophages within atherosclerotic plaques. Recently, several reports demonstrated that CRP could modulate endothelial functions and leukocyte activities. CRP also induces the production of IL-1α, IL-1β, IL-6, CXCL1, and CXCL8 by human monocytes *in vitro*. In contrast to these proinflammatory properties, CRP also displays anti-inflammatory effects through the upregulation of liver X receptor-α [122]. CRP binds to minimally modified (mm) LDL to prevent the foam cell formation from macrophages [123]. Based on animal experiments and the cardiovascular risk stratification in primary prevention populations, the Centers for Disease Control and Prevention and the American Heart Association assigned CRP as an independent marker of cardiovascular risk. The recommended cut-off points in clinical practice are 1 mg/L for low-risk and 3 mg/L for high-risk individuals [124].

4.7. Superoxide Production: Reactive Oxygen Species (ROS). Extensive ROS has been implicated in atherosclerosis by inducing the chronic activation of vascular endothelium and components of immune systems. It has been demonstrated that superoxide production from both macrophages and vascular cells plays a critical role in atherogenesis [125]. When ROS production exceeds the scavenging capacity of cellular antioxidant systems, the resulting oxidative stresses damage lipids, membranes, proteins, and DNAs.

4.8. Emerging Future Biomarkers: MicroRNAs (miRNAs). miRNAs are highly conserved single-stranded noncoding small RNAs that control cellular functions by either degrading mRNAs or inhibiting their translation [126]. The involvement of miRNAs in different aspects of cardiovascular diseases has emerged as an important research field. The dysregulation of many individual miRNAs has been linked to the development and progression of cardiovascular diseases. The forced expression or suppression of a single miRNA is enough to cause or alleviate pathological changes. The roles of miRNAs in the pathogenesis of heart and vascular diseases suggest the possibility of using miRNAs as a potential diagnostic biomarker and/or therapeutic target for cardiovascular diseases [127].

As previously discussed, a critical step in the development of chronic inflammatory atherosclerotic diseases is the migration of circulating monocytes into the subendothelial space and their differentiation into macrophages. A recent study showed that miR-125a-5p mediates lipid uptake and decreases the secretion of some inflammatory cytokines, including IL-2, IL-6, TNF-α, and TGF-β from oxLDL-stimulated monocyte-derived macrophages [128]. The target gene of miR-125a-5p has been found to be ORP9, which has diverse roles in the regulation of lipid metabolism, including vesicle transport, and cell cycle regulation and differentiation [129]. miR-33 appears to regulate both HDL biogenesis in the liver and cellular cholesterol efflux [130]. miR-33 is an intronic miRNA located within the gene encoding sterol-regulatory element-binding factor-2, a transcriptional regulator of cholesterol synthesis. miR-33 modulates the expression of genes that are involved in cellular cholesterol transport. It appears to be regulated by dietary cholesterol *in vivo* and have several roles in cholesterol homeostasis [131]. miR-33 targets the 3′ UTR of ABCA1 in mouse peritoneal macrophages and human cells [131, 132], resulting in reduced atherogenic cholesterol efflux to apolipoprotein A1. Similarly, in a mouse model, the lentiviral delivery of miR-33 represses ABCA1 expression in the liver, leading to a reduction in circulating HDL levels, whereas mice expressing anti-miR-33 demonstrate increased plasma HDL levels [132]. Clearly, miR-33 is a promising target for the treatment of abnormalities in lipoprotein metabolism that frequently contributes to atherosclerosis.

5. Conclusion

The accumulation of macrophages laden with cholesterol in the vascular intima is the hallmark of fatty plaque formation in atherosclerosis. Understanding the mechanisms involving macrophages is critical for the prognosis, diagnosis, and treatment of atherosclerosis. However, because most papers cited in this paper show data from cultured macrophages and animal models, these data may not completely reflect the process in human diseases. As noted by Rosenfeld et al., mouse atherosclerosis is not a good model for true plaque rupture or thrombosis [133]. In contrast, some papers on atherosclerosis emphasise the fact that many genes involved in macrophages have "major and critical" functions for plaques, which complicates the process of determining useful biomarkers for atherosclerosis. Human genetic studies and mechanism-based clinical trials should be performed in the future.

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Review Article

Thrombopoietin as Biomarker and Mediator of Cardiovascular Damage in Critical Diseases

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Thrombopoietin (TPO) is a humoral growth factor originally identified for its ability to stimulate the proliferation and differentiation of megakaryocytes. In addition to its actions on thrombopoiesis, TPO directly modulates the homeostatic potential of mature platelets by influencing their response to several stimuli. In particular, TPO does not induce platelet aggregation *per se* but is able to enhance platelet aggregation in response to different agonists ("priming effect"). Our research group was actively involved, in the last years, in characterizing the effects of TPO in several human critical diseases. In particular, we found that TPO enhances platelet activation and monocyte-platelet interaction in patients with unstable angina, chronic cigarette smokers, and patients with burn injury and burn injury complicated with sepsis. Moreover, we showed that TPO negatively modulates myocardial contractility by stimulating its receptor c-Mpl on cardiomyocytes and the subsequent production of NO, and it mediates the cardiodepressant activity exerted *in vitro* by serum of septic shock patients by cooperating with TNF-α and IL-1β. This paper will summarize the most recent results obtained by our research group on the pathogenic role of elevated TPO levels in these diseases and discuss them together with other recently published important studies on this topic.

1. Introduction

Thrombopoietin (TPO) is a humoral growth factor originally identified using antisense oligonucleotides to c-mpl [1], a protooncogene that is the human homolog of *v-mpl*, the viral oncogene responsible for the transforming myeloproliferative leukemia virus (MPLV) [2]. Initial characterization of c-Mpl-deficient mice showed that they have severe thrombocytopenia but relatively normal levels of other hematological cell types [3]. Subsequently, several research groups identified TPO as the primary ligand for the c-Mpl receptor [4–10] and TPO/Mpl signal transduction was shown to play critical roles in thrombopoiesis, from *ex vivo* megakaryocyte progenitor expansion and differentiation to *in vivo* platelet production [4–11].

TPO is constitutively produced in the liver and kidneys and is then cleared from circulation upon binding with its receptor c-Mpl [7, 8]. Upon TPO binding, c-Mpl receptors undergo homodimerization to initiate intracellular signaling, including activation of the JAK2/signal transducers and activators of transcription (STAT) pathway [12].

In addition to its role in thrombopoiesis, TPO also plays a role in expanding erythroid and granulocytic-monocytic progenitors [13], and loss of TPO/Mpl signaling was associated to a potential defect in the multipotent cell compartment or even in the stem cell compartment [14, 15]. Subsequent studies clearly defined the role of TPO in expanding or maintaining the pool of transplantable hematopoietic stem cells, further establishing the responsiveness of cells in the primitive hematopoietic compartment to this cytokine signaling pathway [16, 17].

Elevated plasma TPO levels have been reported in different clinical conditions, including several hematological diseases usually associated with thrombocytopenia, where increased circulating TPO may be a response to altered bone marrow hematopoiesis or bone marrow failure [18–20]. Of

Table 1: List of publications describing biological functions of TPO/c-MPL system apart from hematopoietic mechanisms.

Mature platelets	[12, 25, 30–32, 64, 72, 109–118]
Polymorphonuclear leukocytes	[86, 119, 120]
Endothelial cells	[96–99, 121, 122]
Cardiac cells	[85, 100–102]
Brain cells	[70, 123–127]
Ovarian cells	[128]
Cancer cells	[129, 130]

particular importance is the example of immune thrombocytopenia, a disorder characterized by immune-mediated platelet destruction and impaired platelet production, resulting in platelet count lower than 100,000 per cubic millimeter and varying degrees of bleeding risk [21]. In patients with immune thrombocytopenia, indeed, TPO levels are usually normal or only slightly increased for reasons that remain unclear [19, 22]. This observation led to the concept of treating the disorder by means of exogenous stimulation of TPO receptors and to the development of TPO-receptor agonists, whose clinical use was recently approved for adult patients affected by immune thrombocytopenia at risk for bleeding [21, 23].

Besides hematological diseases, elevated circulating TPO levels have been also reported in other clinical conditions, including critical diseases such as acute coronary syndromes [24, 25] and sepsis [26–29] (Table 1). This paper will summarize the most recent results obtained by our research group on the pathogenic role of elevated TPO levels in these diseases and discuss them together with other recently published important studies on this topic.

2. Effects of Thrombopoietin on Platelet Activation

Shortly after the cloning and characterization of TPO as c-Mpl ligand, we and others have shown that the TPO receptors are expressed by mature platelets and that TPO directly modulates the homeostatic potential of platelets by influencing their response to several stimuli [30, 31]. In particular TPO does not induce platelet aggregation *per se*, both in platelet-rich plasma and in whole blood, but is able to enhance platelet aggregation in response to different agonists ("priming effect") [30, 31]. In addition, TPO stimulates platelet-leukocyte associations in whole blood through expression of platelet P-selectin [32]. The characterization of this "priming effect" prompted us to study the pathophysiological effects of plasma TPO in those clinical conditions where TPO levels are increased and platelet activation has a pathogenic role.

3. Thrombopoietin Increases Platelet Activation in Patients with Unstable Angina

It is known that patients with unstable angina (UA) have hypersensitive platelets, increased levels of circulating platelet aggregates and platelet secretory products [33–37]; they also show increased platelet-leukocyte aggregates, which have been proposed to represent a better reflection of plaque instability and ongoing vascular thrombosis and inflammation [38, 39]. Activated platelets deposit indeed at sites of unstable plaque rupture and may potentiate thrombus formation, precipitating or exacerbating coronary vascular obstruction [35]. The clinical efficacy of antiplatelet therapies also confirms the importance of platelets in acute coronary syndromes [40]; however, their incomplete effectiveness suggests that alternative platelet activation pathways may be important [40].

Higher levels of TPO were previously reported by Senaran and colleagues [24] in patients with acute coronary syndromes than control subjects and shown to correlate with platelet size, thus potentially resulting in hemostatically more active platelets [24].

In our study [25], we enrolled 15 patients with unstable angina (UA) and, as controls, 15 patients with stable angina (SA) and 15 healthy subjects. We measured circulating levels of TPO by ELISA and of C-reactive protein (CRP) by immunoturbidimetric assay, as well as *ex vivo* monocyte-platelet binding and the platelet expression of P-selectin and of the TPO receptor, c-Mpl, by flow cytometry [41]. Finally, the priming activity of patient or control plasma on platelet aggregation and monocyte-platelet binding [25] and the role of TPO in this effect were also studied *in vitro*.

Confirming what was previously reported by others [24], we found higher circulating TPO levels in patients with UA, at the time of hospital admission, than in patients with SA or healthy controls [25]. Of note, we enrolled the UA patients in the emergency room, before any therapeutic intervention was started, and we therefore believe that our data may closely reflect the ongoing pathogenic events leading to the development of UA.

Elevation of TPO in the plasma of patients with UA was also indirectly confirmed by the finding of reduced surface expression of c-Mpl on circulating platelets, whereas no such a change was detected in patients with SA or healthy controls [25]. Receptor-mediated internalization is indeed considered the primary mean of regulating plasma TPO levels [42, 43]. Diminished c-Mpl expression in platelets from UA patients may thus depend on the previous binding of TPO to its receptor *in vivo*, followed by its internalization and surface downregulation [42, 43]. In addition, the *in vitro* stimulation with recombinant human TPO (rhTPO) was able to phosphorylate c-Mpl only in platelets from SA patients and healthy controls, but not in those from UA patients, further sustaining this hypothesis [25]. This same mechanism was previously shown for thrombocytopenic mice [44] and humans with the syndrome of congenital thrombocytopenia with absent radii [45], whose platelets are exposed, *in vivo* to increased levels of endogenous TPO.

Decreased surface levels of c-Mpl together with decreased platelet sensitivity to exogenous stimulation *in vitro* with a pegylated N-terminal domain of human TPO (PEG-rHuMGDF) have also been evoked as a "protective" mechanism against the prothrombotic effects of *in vivo* treatment with PEG-rHuMGDF in a rat model of mesenteric

microthrombosis [46]; however, the conclusions of this study refer only to thrombocytopenic states, in which endogenous TPO levels are elevated [44–46], whereas this and our subsequent studies were addressed to specific pathologic conditions in which TPO levels rise in presence of normal platelet counts. The precise origin of the rise in plasma TPO level in UA patients remains unclear. We found that CRP levels were increased in this study group, suggesting that the liver acute-phase response, which takes place in acute coronary syndromes [47–49], may have a role in increasing TPO levels. Considering the evidence that elevated CRP has independent prognostic value in UA [50, 51], it is tempting to speculate that the negative prognostic implications of high CRP levels in patients with UA may be at least partially related to the concomitant increase in TPO production and subsequent priming of platelet aggregation. However, activated platelets could also represent a major contributor to the elevated TPO levels observed in UA patients, since they are known to release full-length biological active TPO upon stimulation [52]. In addition, platelet alpha-granular proteins may increase TPO gene expression and consequent TPO production in bone marrow stromal cells via a feed-back mechanism [53, 54].

In addition to elevated plasmatic concentrations of TPO, patients with UA also showed increased indexes of *ex vivo* platelet activation, such as monocyte-platelet binding and platelet P-selectin expression [25].

The presence of TPO in the circulation precludes the evaluation of its role on platelet aggregation directly on blood samples obtained from patients. Therefore, we studied the contribution of TPO to platelet aggregation by adding patient plasma samples to platelets of healthy subjects *in vitro* and inhibiting TPO biological activity by using a TPOR-Fc chimera synthesized in our laboratory [25]. In these experimental conditions, plasma from patients with UA, but not from SA patients or healthy controls, markedly enhances platelet aggregation as well as monocyte-platelet binding in blood samples from healthy donors, stressing the importance of elevated TPO concentrations in the pathogenesis of increased platelet aggregation in UA [25]. Several data show that the "priming effect" exerted by plasma samples from patients with UA may be due to their content in TPO: (1) the priming effect induced by plasma from UA patients was significantly decreased when TPO activity was inhibited with the TPOR-Fc chimera; and (2) adjusting the concentrations of TPO in plasma from SA patients to those measured in UA patients by adding exogenous rhTPO induced a significant increase of the priming effect, similar to that observed with plasma from patients with UA, on ADP- or EPI-induced aggregation in PRP. *In vivo*, a similar "priming effect" induced by TPO on platelet activation has also been documented in nonhuman primates; platelets derived from TPO-treated animals showed indeed a heightened sensitivity to substances that stimulate platelet aggregation during the first few days of treatment [55].

Plasma from UA patients also induced a significant priming effect on platelet aggregation in the presence of acetylsalicylic acid or in PRP from healthy subjects after one-week oral acetylsalicylic acid treatment [25]. These results suggest that the activation pathway triggered by TPO is only partially affected by the antiplatelet therapy commonly used in patients with myocardial ischemia [40] and that platelet priming by TPO may represent a mechanism leading to therapeutic failure of antiplatelet agents. Moreover, the phenomenon we described in this study may provide the rationale for more aggressive (double or triple) antiplatelet treatment in patients with acute coronary syndromes.

In conclusion, in this study we showed that elevated levels of circulating TPO may enhance platelet activation and monocyte-platelet interaction in the early phases of UA [25]. Our *ex vivo* and *in vitro* findings suggest an important link between circulating TPO level and the pro-inflammatory and prothrombotic state that occurs in UA patients and implicate TPO in the pathogenesis of acute coronary syndromes, where it could potentially precipitate conditions of clinical instability.

4. Thrombopoietin Increases Platelet Activation in Cigarette Smokers

Chronic smoking is a major risk factor for the development of atherosclerosis and thrombosis, and it has been strongly associated with adverse cardiovascular effects [56]. In particular, enhanced platelet aggregability and subsequent alterations in the clotting cascade have been evoked [57, 58] as main pathogenic factors sustaining the increased risk of coronary artery thrombosis in long-term smokers [57, 59]. Since inflammation plays a central role in the pathogenesis of atherosclerosis and its complications [60], a number of studies have investigated the association between smoking and increase in several inflammatory markers, such as CRP [61], interleukin (IL)-6 [62], tumor necrosis factor (TNF)-α [62], and CD40L [63]. However, the biological mechanisms linking smoking and atherosclerosis are complex and have not been fully elucidated.

In our study [64], we evaluated TPO and CRP levels, platelet-leukocyte binding and the platelet expression of P-selectin, and the priming activity of smoker or control plasma on *in vitro* platelet-monocyte binding in 20 healthy cigarette smokers and 20 age- and gender-matched nonsmokers. In a second phase of the study we investigated the effects of acute smoking and of smoking cessation on TPO levels and *ex vivo* platelet activation markers [64]. For this purpose, healthy non-smoking subjects were studied at the baseline and after they smoked two cigarettes in 30 minutes. Moreover, 8 healthy cigarette smokers were studied at the baseline and after they had quit smoking for three weeks.

In the first part of this study, we found that chronic smokers have higher circulating TPO levels than nonsmokers and higher platelet-leukocyte binding and platelet P-selectin expression *ex vivo* [64]. Although cigarette smokers had significantly higher TPO levels than nonsmokers, the concentrations measured in both groups were lower than those measured in patients with UA [25] and, may someway be considered within the "physiological" range (i.e., in our experience, below 40 pg/mL).

In addition to elevated plasmatic concentrations of TPO, cigarette smokers also showed increased indices of *ex vivo* platelet activation, such as higher platelet-monocyte binding and platelet P-selectin expression [64]. Moreover, TPO levels correlated with *ex vivo* platelet-monocyte aggregation and P-selectin expression [64].

In vitro, plasma from cigarette smokers, but not from nonsmoking subjects, significantly enhanced platelet-monocyte binding in blood samples from healthy donors [64]. The contribution of TPO to this priming effect is suggested by the correlation analysis showing that TPO levels and platelet-monocyte adhesion in whole blood consensually increased in the two groups. Moreover, the direct proof that TPO participates to the platelet activation observed in chronic smokers is provided by the inhibitory effect of the TPOR-Fc chimera [64]. Taken together, our data support the hypothesis that circulating TPO may facilitate platelet activation in smokers by sensitizing platelets to the action of other agonists. Therefore, it can be suggested that TPO is required, but is not sufficient *per se* to promote aggregate formation, and cooperates with other mediators to induce the observed changes on platelet activation in chronic smokers. Interestingly, TPO primes platelet-monocyte binding in chronic smokers even in the presence of increased levels of CRP, which is known to inhibit platelet aggregation and platelet capture of leukocytes [65]. On the contrary, plasma samples from cigarette smokers did not induce a significant priming effect on platelet aggregation both in PRP and whole blood. This discrepancy with the results obtained using flow cytometry may depend on the different sensitivity of these techniques.

Since smoking may also "acutely" affect platelet function [66], we evaluated whether "acute" smoking (two cigarettes in the previous 30 minutes) was able to induce an increase in TPO levels and platelet activation. We found that the changes in TPO levels upon "acute" smoking, in our experimental conditions, were about a half of the increase observed in chronic smokers compared with non-smoking controls [64]. However, this TPO elevation did not affect *ex vivo* platelet activation, evaluated in terms of platelet-leukocyte adhesion and P-selectin expression [64]. These results suggest that transient platelet activation and consequent TPO release by activated platelets induced by "acute" smoking have only a negligible role in causing the increase in platelet activation markers observed in long-term smokers. In addition, "acute" smoking plasma failed to promote EPI-induced platelet-leukocyte binding and P-selectin expression in *in vitro* experiments [64]. This result can be due to several reasons: (a) the short exposure of the subjects to cigarette smoking (all of them were not usual smokers), (b) the relatively low amounts of TPO released after "acute" smoking, which are further diluted in the *in vitro* experiments, and also (c) the lack of the concomitant presence of other factors which can be released after chronic exposure to smoking and cooperate with TPO in enhancing platelet activation.

Alternatively, higher TPO levels in chronic smokers may depend on increased hepatic synthesis, sustained by the liver acute-phase response that takes place in inflammatory diseases, including atherosclerosis [60]. It is known that IL-6, the main acute-phase reactant produced in the liver, enhances TPO synthesis [67]. The findings that chronic smokers have elevated CRP concentrations and that CRP increases concomitantly with TPO would be in agreement with this hypothesis. However, in our study, we were not able to document a concomitant decrease in TPO and CRP concentrations after smoking cessation [64], and we have no direct data sustaining the hepatic origin of TPO in our subjects. Therefore, the precise origin of the rise in plasma TPO level observed in cigarette smokers remains unclear.

Further stressing the importance of chronic exposure to smoking products in the genesis of platelet function abnormalities, chronic cigarette smokers who quit smoking for three weeks showed a significant decrease in TPO levels, which was associated with reduced platelet-monocyte and platelet-granulocyte bindings [64]. Consistently, plasma drawn after smoking cessation induced a significantly lower platelet-monocyte aggregation *in vitro* compared to that induced by plasma obtained before smoking cessation [64]. On the contrary, no differences in platelet-granulocyte adhesion and P-selectin expression were observed [64].

In conclusion, the results of this study suggest that TPO may contribute to enhance platelet activation and platelet-monocyte cross-talk in cigarette smokers and that increased TPO may represent a novel pathogenic mechanism whereby cigarette smoking promotes atherogenesis and is associated with the development of adverse cardiovascular events [64].

5. Thrombopoietin Increases Platelet Activation in Patients with Burn Injury and Burn Injury Complicated with Sepsis

Several investigations show that dysregulation of the TPO/Mpl receptor system is also present in sepsis. In particular, elevated TPO levels have been reported in healthy volunteers after endotoxin infusion [28], as well as in septic children and neonates [26, 68–71] and septic adult patients [27, 29]. More recently, Zakynthinos and colleagues showed that TPO levels are greatly increased in patients with sepsis compared to control subjects and correlate with sepsis severity and that sepsis severity represents the major determinant of elevated TPO levels in these patients [29]. To investigate the potential contribution of elevated TPO levels in platelet activation during burn injury complicated or not by sepsis, we studied 22 burned patients, 10 without and 12 with sepsis, and 10 healthy subjects [72]. We measured plasma levels of TPO, as well as leukocyte-platelet binding and P-selectin expression *in vivo*, and assessed the "priming" activity of plasma from burned patients or healthy subjects on platelet aggregation and leukocyte-platelet binding and the involvement of TPO in these effects *in vitro* [72].

In this study we found that burn injury is associated with a significant increase in the circulating levels of TPO, about twofold the levels measured in healthy subjects [72]. TPO levels further increase upon development of sepsis, suggesting that the development of sepsis, in addition to burn injury,

may contribute to increase circulating TPO levels in these patients [72]. These results are substantially in agreement with those already reported by Zakynthinos and colleagues in a larger population of patients with sepsis [29].

We also found that patients with burn injury show increased monocyte-platelet aggregates and platelet P-selectin expression, compared to healthy subjects [72]. In addition, monocyte-platelet aggregates were significantly higher in burned patients with sepsis than burned patients without sepsis [72]. These findings showed that increased platelet activation (i.e., P-selectin expression) and heterotypic aggregation (i.e., monocyte-platelet adhesion) also occur in burn injury, especially after sepsis development, suggesting that activated platelets amplifies the inflammatory reactions and favor the insurgence of organ damage in these pathological conditions.

The precise origin of the rise in TPO levels observed in burned patients without and with sepsis remains unclear. TPO levels are well known to be primarily regulated by platelet mass [73–75], and yet we did not detect thrombocytopenia in septic patients in our study. Burned patients without and with sepsis also showed increased indices of *in vivo* platelet activation compared to healthy subjects. Moreover, we found a positive correlation between (a) TPO levels and monocyte-platelet binding *in vivo* and (b) TPO levels and platelet P-selectin expression *in vivo*. Therefore, platelets themselves may represent a major contributor to increased TPO levels, or high TPO levels in burned and septic patients may depend on increased hepatic synthesis.

Analogously to previous studies, we studied the contribution of TPO to platelet aggregation by adding patient plasma samples to platelets of healthy subjects *in vitro* and inhibiting TPO biological activity by using the TPOR-Fc chimera [72]. In these experimental conditions, plasma from burned patients without and with sepsis, but not from healthy subjects, enhances platelet aggregation as well as monocyte-platelet binding and platelet P-selectin expression in blood samples from healthy donors [72]. The "priming effect" induced by plasma from burned patients with sepsis was significantly higher than that induced by plasma from burned patients without sepsis in all the experimental conditions tested [72]. The contribution of TPO to the "priming effect" exerted by plasma samples from burned patients without and with sepsis is suggested by (1) the correlation analysis showing that TPO levels and ADP- and EPI-induced priming index in PRP and whole blood consensually increased in the three groups; (2) the inhibitory effect of the TPOR-Fc chimera [72]. Taken together, our *in vivo* and *in vitro* data support the hypothesis that TPO present in the circulation of burned patients, especially those developing sepsis, may facilitate platelet activation by sensitizing circulating platelets to the action of other agonists, thus precipitating the occurrence of microvascular thrombosis and the clinical onset of multiorgan failure.

In conclusion, increased TPO levels may enhance platelet activation during burn injury and sepsis and have a role in the pathogenesis of multiorgan failure in these pathological conditions.

6. Effects of Thrombopoietin on Myocardial Cell Contractility and Implications for Cardiac Dysfunction in Patients with Septic Shock

Myocardial dysfunction is common in patients with sepsis and is associated with high risk to develop multiorgan failure and high mortality rate [76–78]. Septic cardiomyopathy is characterized by reversible biventricular dilatation, decreased ejection fraction, and impaired response to fluid resuscitation and catecholamine stimulation [76–78]. Although also intrinsic cardiac factors have been implicated in this complex condition [76–78], the causal role of circulating factors has been extensively studied [77–79], following the observation that serum from patients with septic shock decreases myocyte contractile function and that this effect correlates with the reduction of the patient's left ventricular ejection fraction [80]. A pivotal role for TNF-α and IL-1β in mediating this depressant activity has clearly emerged from subsequent studies [81]. Each individual cytokine, although at supraphysiological concentrations, as well as the combination of the two at concentrations similar to those measured in the bloodstream of septic patients, is able to reproduce *in vitro* the depressant effect of septic serum [82]. This response is mainly mediated by the production of nitric oxide (NO) and cyclic guanosine monophosphate (cGMP) [83], although also NO-independent mechanisms have been involved [84].

No data on the potential contribution of TPO to myocardial depression during sepsis was available at the time. We therefore planned an experimental *in vitro* study to investigate whether TPO affects myocardial contractile function and contributes to the myocardial depressing activity of septic shock serum [85].

A first original result of this study was the demonstration of the presence of the TPO-receptor c-Mpl in the rat heart by RT-PCR and immunoblotting and in human myocardium by immunoblotting [85]. Since endothelial cells express c-Mpl [7], we studied the presence of c-Mpl also in cultured cardiomyocytes, definitely showing that they express c-Mpl on the cellular surface, as also confirmed by confocal microscopy [85].

We then evaluated the effects of TPO on myocardial contractility *in vitro* and found that TPO did not directly modify the contractile force of isolated rat papillary muscle, but blunted the enhancement of contractile force induced by epinephrine (EPI) in both papillary muscle and isolated heart preparations [85]. We previously observed similar TPO effects in other experimental settings, where TPO exerts no direct action *per se*, but rather amplifies the effects of other biological mediators, in particular when we studied platelet aggregation and monocyte-platelet interaction in response to ADP and EPI [30], or the production of oxygen free radicals by polymorphonuclear leukocytes challenged with fMLP [86].

The specificity of TPO effect was assessed by inhibiting the biological activity of TPO using a TPOR-Fc chimera, which completely abrogated the effects exerted by TPO on

EPI-induced cardiac contractility, whereas it did neither alter the contractile force of papillary muscle or isolated heart nor influence their responsiveness to beta-adrenergic stimulation [85].

The occurrence of myocardial depression in septic shock is a well-documented phenomenon, which is associated with high mortality rate [76–78]. Interestingly, the major pathogenic mechanisms of septic cardiac dysfunction include both alterations of adrenergic response and the effects of humoral mediators [76–79]. Myocardial hyporesponsiveness to catecholamines (including decreased chronotropy and inotropy) has been shown in several endotoxic models of septic shock [87, 88], as well as in human septic shock [89, 90]. This effect has been related to disruption of β-adrenergic signal transduction in cardiomyocytes due to both NO-dependent and -independent mechanisms [84], analogously to what was reported for ischemia/reperfusion injury [91–93]. Our results suggest that also TPO may influence the contractile response elicited by EPI stimulation by affecting adrenergic signal transduction [85]. Moreover, the results obtained by using specific pharmacological inhibitors on isolated rat papillary muscles show that TPO action is mediated by the activation of the PI3K-Akt1-NO Synthase-Guanylyl Cyclase pathway, which leads to the production of NO as final mediator [85]. We have also shown that TPO directly induces the phosphorylation of Akt1 in H9C2 cardiomyocytes and isolated papillary muscles [85].

Evidence of a circulating myocardial depressant substance in the serum of septic shock patients was first demonstrated by Parrillo et al. [80]. Subsequent studies indicated TNF-α and IL-1β as the pivotal mediators inducing cardiac depression in sepsis [82] and other disease conditions [94, 95]. On the basis of the recent reports of elevated TPO levels in sepsis [26–29, 68–72], we hypothesized that TPO may concur to depress myocardial contractility during sepsis. We observed that pretreatment of serum samples with the TPOR-Fc chimera completely prevented the decrease in contractile force-induced by human sepsis shock serum alone, whereas it had no effect on serum of healthy subjects [85]. In addition, we found that the negative inotropic effect of both TNF-α and IL-1β was significantly enhanced by the addition of TPO [85]. Moreover, TPOR-Fc chimera completely prevented the decrease in contractile force induced by addition of TPO to TNF-α or IL-1β, whereas it did not modify the contractile responses induced by these cytokines alone [85]. The results obtained strongly suggest that high levels of circulating TPO in patients with sepsis may favor the occurrence of myocardial depression in cooperation with TNF-α and IL-1β.

In conclusion, we showed in this study that TPO negatively modulates myocardial contractility by stimulating its receptor c-Mpl on cardiomyocytes and the subsequent production of NO [85]. In addition, TPO mediates the cardiodepressant activity exerted *in vitro* by serum of septic shock patients, which is indeed completely abrogated by the TPOR-Fc chimera [85]. Finally, TPO cooperates with TNF-α and IL-1β in depressing cardiac contractility [85]. Therefore, our results suggest that TPO may have a relevant role in modulating cardiac inotropy in septic shock by affecting the two major pathogenic mechanisms described: (a) by influencing adrenergic receptor signal transduction; (b) by cooperating with circulating mediators known to reduce myocardial contractility, namely TNF-α and IL-1β.

7. Characterization of Thrombopoietin as a Physiological Regulator of Coronary Flow

We have previously shown that human umbilical cord vein-derived endothelial cells (HUVECs) expressed the TPO receptor c-Mpl and that TPO activates HUVECs *in vitro*, as indicated by directional migration, synthesis of platelet-activating factor and IL-8, and phosphorylation of STAT1 and STAT5B [96]. Others have shown that specific murine liver EC (LEC-1) located in the hepatic sinusoids coexpresses TPO and its receptor, c-Mp, and that TPO has a proliferative effect on LEC-1 [97]. Furthermore, stimulation with TPO induced secretion of proinflammatory cytokines (i.e., IL-1β, IL-6, TNF-α) from LEC-1, some of which cooperate with TPO in sustaining the proliferation of these cells [98].

Based on these data, we sought to investigate the potential role of TPO in coronary flow modulation and to determine the mechanisms involved.

The expression of TPO receptor c-Mpl and the TPO-dependent eNOS phosphorylation ($P^{Ser1179}$) were showed on cardiac-derived normal human microvascular endothelial cells (HMVEC-C) by Western blot analysis [99]. While TPO (10–200 pg/mL) did not modify coronary flow (CF) under basal conditions, it reduced the coronary constriction caused by endothelin-1 (ET-1; 10 nM) in a dose-dependent manner [99]. This effect was blocked by both Wortmannin (100 nM) and L-NAME (100 nM); on HMVEC-C, TPO induced eNOS phosphorylation through a Wortmannin sensitive mechanism [99]. Our data suggest a potential role of TPO as a physiological regulator of CF. By acting on specific receptors present on endothelial cells, TPO may induce PI3K/Akt-dependent eNOS phosphorylation and NO release.

8. Effects of Thrombopoietin Treatment on Myocardial Cell Viability and Cardiac Function in Experimental Models of Myocardial Ischaemia-Reperfusion Injury and Heart Failure

Recent experimental studies by other groups reported results apparently not consistent with ours [100–102].

Li and colleagues [100], based on the rationale that TPO possesses antiapoptotic functions mediated by the Akt pro-survival axis in hematopoietic stem cells and megakaryocytes [103, 104], hypothesized that TPO may protect against cardiotoxicity induced by doxorubicin. In their study, they showed that TPO exerts antiapoptotic activity in two different *in vitro* cellular models, namely, the fetal rat cardiomyocyte cell line H9C2 and spontaneously beating primary neonatal rat cardiomyocytes [100]. Moreover, TPO was able to preserve cardiac functions, including heart rate, fractional shortening, and cardiac output, evaluated by echocardiography, in

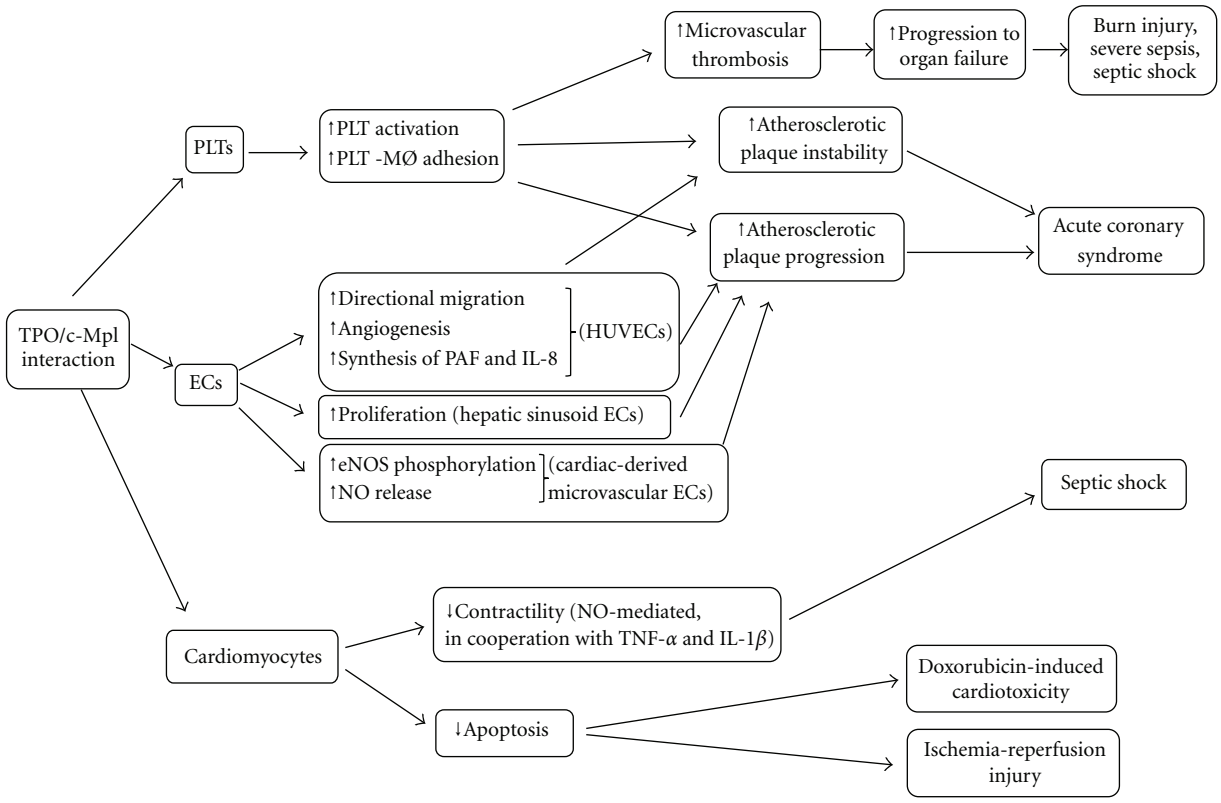

Figure 1: Schematic representation of TPO/c-Mpl functions in vascular system. PLTs = platelets; ECs = endothelial cells; MØ = monocytes; PAF = platelet-activating factor; IL-8 = interleukin-8; HUVECs = human umbilical vein-derived endothelial cells; NO = nitric oxide; TNF-α = tumor necrosis factor-α; IL-1β = interleukin-1β.

an *in vivo* model of doxorubicin-induced acute cardiotoxicity [100].

More recently, the same research group extended their previous results by studying cardiac damage in two different rat models of acute- and chronic-doxorubicin treatment [102]. In both models TPO treatment led to significant improvements of fractional shortening, cardiac output, and morphologic parameters [102]. In the acute-doxorubicin model, microarray and network analyses showed that cardiac damage was associated with changes in a large cohort of gene expressions, of which many were inversely regulated by TPO, including modulators of signal transduction, ion transport, antiapoptosis, protein kinase B/p42/p44 extracellular signal-regulated kinase (AKT/ERK) pathways, cell division, and contractile protein/matrix remodeling [102]. Many of these regulations also occurred in the animals chronically treated with doxorubicin, in which TPO treatment reduced morphological damage and cardiomyopathy score, and increased AKT phosphorylation of heart tissues [102]. TPO was also shown to increase the formation of endothelial progenitor cell (EPC) colonies in their bone marrow [102]. The conclusions of the authors suggest that TPO-induced cardiac protection from acute- and chronic-doxorubicin damages may be mediated by multifactorial mechanisms including AKT- and ERK-associated restoration of regulatory gene activities critical for normal heart function [102].

In another recent study, Baker and colleagues demonstrated that TPO treatment immediately before ischaemia reduced myocardial necrosis, apoptosis, and decline in ventricular function following ischaemia/reperfusion both *in vitro* in the rat isolated heart and *in vivo* [101]. This TPO effect is concentration and dose dependent with an optimal concentration of 1.0 ng/mL *in vitro* and an optimal dose of 0.05 μg/kg iv *in vivo* [101]. Increased resistance to injury from myocardial ischaemia/reperfusion conferred by TPO has been shown to be mediated by JAK-2, p42/44 MAPK, and K_{ATP} channels [101].

9. Discussion and Conclusions

We have no clear explanation for the discrepancy between the results summarised in the last paragraph [100–102] and ours [85], although the different experimental models, together with the concentrations used, well above the physiological range, may, at least partially, justify these results.

In our study [85] we evaluated indeed TPO cardiac effects at doses analogous to those measured in human pathology, in particular during septic shock [29], whereas, for instance, Li and colleagues used TPO concentrations as high as 50 ng/mL or 100 ng/mL [100], which are commonly reported as effective in promoting hematopoietic cells in culture [105, 106]. Moreover, in our study [85] we focused on the

acute changes induced by TPO pretreatment on EPI-stimulated myocardial contractility in a time frame in which apoptosis could difficultly take place, thus making it difficult to compare ours with the results published by others. More generally, all these studies were performed in rodent *in vivo* models; therefore we have to be cautious in the eventual transfer of the results to the pathophysiology of human diseases. For instance, the experimental model of doxorubicin-induced acute cardiotoxicity chosen [107, 108] does not closely mimic the chronic cardiomyopathy observed in clinical situations during which repeated smaller doses of doxorubicin are given over a period of time, as reported by the same authors [100]. Finally, and most important, although it is well known that TPO primes the activity of other mediators on important biological effects in mature platelets and other cell types [30–32, 86], previously reported results were obtained in controlled experimental conditions, in which only the effects of TPO alone and at high doses were considered, while the combined effects of the administration of TPO with other soluble mediators were not evaluated [100–102]. On the contrary, we studied the effects of TPO contained in the plasma samples of patients with different pathological conditions [25, 64, 72, 85], and we were able to show the contribution of TPO to the biological effects studied by selectively inhibiting TPO activity with a TPOR-FC chimeric protein synthesized in our laboratory [25, 64, 72, 85]. Moreover, we investigated the effects of TPO on myocardial contractility at physiologic concentrations and in association either with an adrenergic stimulus (EPI) or the main cytokines known to depress myocardial activity in septic shock, that is, TNF-α and IL-1β [85].

Therefore, our findings stress the importance of careful evaluation of the cardiovascular effects of TPO *in vivo*, especially in the clinical setting of diseases, such as heart failure and septic shock, whose pathogenesis is complex and involves the activation of a cascade of soluble mediators (Figure 1). Interestingly, large-scale clinical trials evaluating the effects of monoclonal antibodies against TNF-α in chronic heart failure patients gave rather disappointing outcomes [131, 132], suggesting that counterbalancing this cytokine alone may not be sufficient [133]. The individuation of TPO as an additional molecular target may provide clue for the development of new therapeutic interventions for the treatment of patients with sepsis-associated cardiac dysfunction and eventually chronic heart failure. Interestingly, several recent studies proposed also new experimental therapeutic approaches to various cardiac diseases based on the modulation of the inflammatory and prothrombotic states which are often associated with their development and progression [134–136]. Moreover, a careful and complete evaluation of the biological effects of TPO *in vivo*, in particular for what concerns cardiovascular consequences, needs to be envisaged.

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Research Article

Mobilization of CD34+CXCR4+ Stem/Progenitor Cells and the Parameters of Left Ventricular Function and Remodeling in 1-Year Follow-up of Patients with Acute Myocardial Infarction

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Mobilization of stem cells in acute MI might signify the reparatory response. *Aim of the Study*. Prospective evaluation of correlation between CD34+CXCR4+ cell mobilization and improvement of LVEF and remodeling in patients with acute MI in 1-year followup. *Methods*. 50 patients with MI, 28 with stable angina (SAP), and 20 individuals with no CAD (CTRL). CD34+CXCR4+ cells, SDF-1, G-CSF, troponin I (TnI) and NT-proBNP were measured on admission and 1 year after MI. Echocardiography and ergospirometry were carried out after 1 year. *Results*. Number of CD34+CXCR4+ cells in acute MI was significantly higher in comparison with SAP and CTRL, but lower in patients with decreased LVEF ≤40%. In patients who had significant LVEF increase ≥5% in 1 year FU the number of cells in acute MI was significantly higher versus patients with no LVEF improvement. Number of cells was positively correlated ($r = 0,41$, $P = 0,031$) with absolute LVEF change and inversely with absolute change of ESD and EDD in 1-year FU. Mobilization of CD34+CXCR4+ cells in acute MI was negatively correlated with maximum TnI and NT-proBNP levels. *Conclusion*. Mobilization of CD34+CXCR4+ cells in acute MI shows significant positive correlation with improvement of LVEF after 1 year.

1. Background

Small numbers of bone-marrow (BM-) derived stem and progenitor cells (SPC) are present in peripheral blood in humans. In acute coronary syndromes (ACS) and stroke the number of circulating cells significantly increases. Such mobilization of SPC is an inflammatory reaction, but the presence of primitive SPC can also reflect the reparatory mechanism. Mobilization of endothelial progenitor cells (EPCs) reflects the turnover of vascular endothelial cells, because these cells contribute to endothelial renewal [1–3]. Myocardial infarction (MI) triggers the mobilization of not only EPCs, but also other populations such as hematopoietic stem cells (HSCs), mesenchymal stromal sells (MSCs), very small embryonic like cells (VSELs) and other less well-defined types [4, 5]. One of the populations that undergoes rapid mobilization in acute MI are cells expressing chemokine receptor CXCR4. These cells are enriched for early markers of myocardial and endothelial differentiation and in part also markers for primitive embryonic-like stem cells (Oct-4, SSEA-4, Nanog) [5]. Our previous studies demonstrated that in acute MI within several hours after the onset of the chest pain there is a robust increase of CD34+CXCR4+ and CD34+CD117+ cells. The mobilization coexists with

significant upregulation of cardiac (GATA-4, Nkx2.5/Csx, MEF2C) and endothelial lineage markers (VE-cadherin, von Willebrand factor), which suggests that these cells might contribute to tissue repair following ischemic injury [4]. Mobilization of BM-derived SPC is regulated by chemoattractants released by ischemic myocardium, complement cascade, and bioactive phospholipids [6].

In particular stromal-derived factor-1 (SDF-1)–CXCR4—axis might contribute to homing of the SPC to the infarct border area in the heart where it is expressed following MI. This signaling axis is also the key factor regulating the mobilization of BM cells and renewal of hematopoiesis as well as in inflammation [7]. Mobilization of BM by G-CSF is mediated by disruption of SDF-1-CXCR4 binding [8]. Increased production of SDF-1 via activation of hypoxia-inducible factor 1-α within the ischemic myocardium facilitates the homing and engraftment of circulating BM cells which subsequently participate in the reparatory processes [9].

Mobilization of SPC was investigated as a potential prognostic marker in patients with stable coronary artery disease (CAD) and the number of circulating EPCs correlated with CAD risk factors, endothelium-dependent vasomotion, and risk of ischemic events [10–12].

Prognostic value of measurement of SPC mobilization in ACS is less well known. Acute MI triggers substantial inflammatory response which might affect the mobilization and trafficking of stem cells. In addition, intensive treatment with drugs known to affect the SPC release from the BM such as statins and ACE-I might modulate to mobilization and migration intensity. Other important factors are patients age and comorbidities in particular diabetes [13]. There is a paucity of data on the association between mobilization of SPC which might contribute to myocardial tissue repair and the improvement of the left ventricle (LV) contractility and remodeling; however, pilot studies showed that in patients with reduced LVEF in acute MI the mobilization of cells is less efficient [14].

Improvement of LVEF following the primary percutaneous coronary intervention (pPCI) is a positive prognostic factor for long-term survival in acute MI. Spontaneous mobilization of SPC in acute MI is a form of reparatory mechanism; therefore we conducted a prospective study to evaluate the relationship of CD34+CXCR4+ cell mobilization and long-term recovery of LV contractility, remodeling, and clinical status (ergospirometry, NYHA, CCS class) in patients with acute MI in 1-year follow-up.

2. Patients and Methods

Study population consisted of 98 patients: 50 patients with acute myocardial infarction (MI), 28 patients with stable angina pectoris (SAP), and 20 individuals with no history of ischemic heart disease (control group, CTRL). Subjects with myocardial infarction were diagnosed according to the current ST-elevation myocardial MI (STEMI) definition.

Inclusion criteria for patients with myocardial infarction were

(1) time interval between the onset of chest pain and hospital admission <12 hours,

(2) age < 75 years,

(3) patients qualified to pPCI.

Abciximab was administered in 64% of patients during PCI procedure. All patients received unfractionated heparin (70 U/kg) to achieve ACT values >250. In all patients TIMI3 flow in the infarct-related artery was achieved. Statins (67% simvastatin and 33% atorvastatin) were administered starting from the first day of hospitalization.

Exclusion criteria were

(1) history of MI in the past,

(2) cardiogenic shock (IV class according to Killip-Kimball scale),

(3) neoplastic disease,

(4) kidney and/or liver failure,

(5) coagulopathies and/or hematopoietic system diseases,

(6) autoimmunological disorder and/or systemic inflammatory process,

(7) history of surgical procedure or coronary arteries percutaneous intervention (revascularization) within last 6 months.

Patients were diagnosed to have stable angina pectoris according to the following: (a) typical clinical presentation/symptoms (chest or arm discomfort/angina reproducibly associated with physical exercise), (b) noninvasive test (positive exercise test/treadmill stress test) and qualified to planned coronarography. Presence of ≥ 1 significant stenotic lesion ($\geq 70\%$) in coronary arteries was reported. Stable angina pectoris (SAP) and acute myocardial infarction (AMI) groups were matched to avoid major differences in the context of risk factors and pharmacological treatment which may affect the number of cells circulating cells.

Control group (CTRL) individuals were diagnosed due to valvular heart disease or rhythm disturbances.

The study protocol was approved by the Ethics Committee of the Medical University of Silesia and all patients signed informed consent. The study conformed to the Declaration of Helsinki and was funded by the European Union structural funds—Innovative Economy Operational Programme, Grant POIG.01.01.02-00-109/09 "Innovative methods of stem cells applications in medicine" and Polish Ministry of Science and Higher Education Grants 0651/P01/2007/32, 2422/P01/2007/32 and statutory funds of Medical University of Silesia.

2.1. Laboratory Measurements. Peripheral blood (PB) samples were collected within 12 hours of the first symptoms and 1 year after in patients with myocardial infarction, in SAP and control group during routine clinical follow-up visit. 4–6 mL of PB was obtained from each patient and stored in

both vacuum heparin tubes (2-3 mL; measurement of progenitor cell number) and vacuum EDTA tubes (2-3 mL; measurement of hematopoietic cytokines concentration).

The following parameters were measured:

(1) number of CD34+/CXCR4+ progenitor cells,

(2) concentration of chemoattractant factors (SDF-1, G-CSF),

(3) troponin I (TnI) concentration and creatine kinase MB isoenzyme (CK-MB) activity,

(4) NT-proBNP and high sensitive C-reactive protein (hsCRP) concentration.

2.1.1. Measurement of CD34+CXCR4+ Cells. Blood samples were transported in 4°C to FACS facility processed within 4–6 hours after drawing. CD34+CXCR4+ cells number was analyzed with FACS based on specific membrane antigens expression in accordance to the ISHAGE criteria (International Society of Hematotherapy and Graft Engineering) [15]. For isolation of mononuclear cells (MNCs) samples were centrifuged through a Ficoll density gradient and subsequently suspended in phosphate-buffered saline (PBS) (1×10^5/100 uL). Afterwards, MNCs were stained with fluorochrome-conjugated mouse monoclonal antibodies (Abs) for the CD34 (phycoerythrin- (PE) conjugated Abs) and CXCR4 (allophycocyanin- [APC-]conjugated Abs) and isotope control (BD, Pharmingen, San Diego, CA, USA). Staining was performed at 4°C for 30 minutes without light exposure. Cells were subsequently washed twice in PBS, resuspended in 200 μL of PBS, and analyzed using a flow cytometer (FACSCalibur, Becton Dickinson, San Jose, USA). At least 106 events were acquired from each sample. The percentage content of CD34+/CXCR4+ cells was calculated with appropriate isotope control cut-offs. The absolute number of (cells/μL) was calculated according to the previously published method: CD34+/CXCR4+ percentage × leucocytes number/100 [14].

2.1.2. Chemoattractant and Inflammatory Markers. Collected PB samples were centrifugated (1000 ×g) at 4°C for 15 minutes. Obtained plasma was stored at −30°C. The centrifugation was performed within 30 minutes from blood sampling. Additionally, for SDF-1 level measurement samples were centrifuged (10 000 ×g) for 10 minutes in order to eliminate platelets. Plasma levels of SDF-1, G-CSF, NT-proBNP, and C-reactive protein were quantified using high sensitive kits (G-CSF (Bender Medsystems); SDF-1 (Quantikine, R&D systems); NT-proBNP (Quantikine, R&D systems), hsCRP (Behring Nephelometer II Dade Behring)).

2.2. Echocardiography. Echocardiography was performed after admission to hospital (<12 hours of chest pain symptoms) and 12 months post discharge during the follow-up visit by experienced echocardiolographer. Transthoracic echocardiography (M-mode and typical 2D projections) was carried out in accordance to the American Society of Echocardiography guidelines.

Evaluated echocardiography parameters were: left ventricle end-diastolic (EDD) and end-systolic (ESD) diameter and left ventricle ejection fraction (EF%) according to Simpson method.

2.3. Ergospirometric Test. The test was performed 12 months after myocardial infarction on the treadmill according to modified Bruce protocol. The following parameters were analyzed: resting heart rate (HRrest), peak heart rate (HRpeak), maximum exercise time (Tmax), energy expenditure in METs, maximal exertional oxygen uptake (VO_2 peak) presented as mL/kg/min and percentage of calculated norm (VO_2 peak %N), resting and peak ventilatory equivalent for oxygen (VE/VO_2 rest, VE/VO_2 peak), peak/resting ventilatory equivalent for oxygen ratio (VE/VO_2 peak/rest), resting and peak ventilatory equivalent for carbon dioxide (VE/VCO_2 rest, VE/VCO_2 peak), peak/resting ventilatory equivalent for carbon dioxide ratio (VE/VCO_2 peak/rest), ventilation relative to carbon dioxide production (VE/VCO_2 slope), oxygen pulse and heart rate reserve (HRR)—according to the American College of Sports Medicine guidelines and methods. Test was continued until limiting symptoms (fatigue, chest pain, dyspnea) or lack of VO2 increase occurred. Test was carried out on Oxycon Delta (Jaeger) system.

2.4. Clinical Status. Heart failure symptoms were evaluated according to New York Heart Association (NYHA) classification and angina severity according to Canadian Cardiovascular Society (CCS) class.

2.5. Statistical Analysis. Number of SPC and levels of chemoattractants were expressed as median and interquartile range (IQR). U Mann-Whitney and Wilcoxon tests were used for comparison of time points and groups and Spearman rank test for assessment of correlation. Logistic regression was used to identify the factors associated with significant (2-fold) mobilization of cells. Value of $P < 0.05$ was considered significant. Statistica 6.0 PL for Windows package was used.

3. Results

Study groups (AMI and SAP patients) were comparable with respect to risk factors profile, demographic data, and laboratory results excluding leucocyte number which was statistically significantly higher in MI group. In comparison to SA group patients with MI less frequently were on chronic treatment with ASA ($n = 31$ (62%) versus $n = 28$ (100%), $P < 0.05$). In MI group anterior MI was diagnosed in 30 (60%) and multivessel coronary disease in 26 patients (52%). In comparison of MI and SAP group with CTRL group the following parameters were statistically significantly higher in the study groups: mean age, percentage of patients with hypertension, hypercholesterolemia, type 2 diabetes mellitus, and family history of ischemic heart disease and smoking. Clinical and demographic characteristics of the study population is shown are Table 1. 50 patients were followedup 1 year after MI. The medical treatment at the time of followup

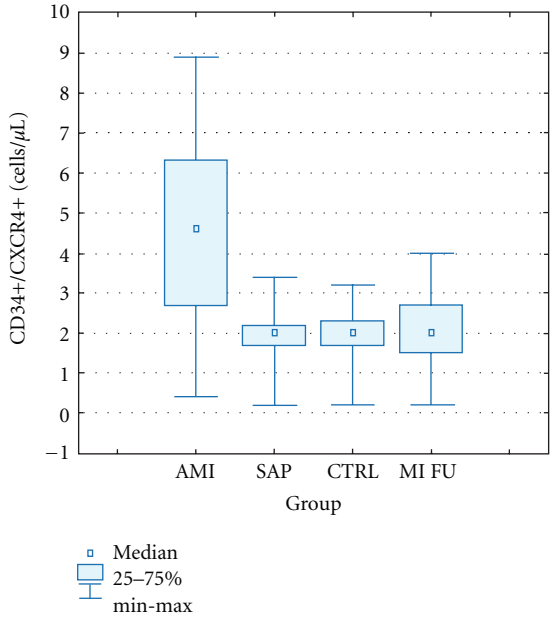

Figure 1: The number of circulating progenitor CD34+/CXCR4+ cells in peripheral blood. AMI: acute myocardial infarction, SAP: stable angina pectoris, CTRL: control group, MI FU: 1-year follow-up. Data is presented as cell number in 1 μL of peripheral blood (median ± IQR). CD34+CXCR4+: CTRL 2,0 (0,2–3,4); SAP 2,1 (0,2–3,2); AMI 4,6 (0,4–8,9); MI FU 2,2 (0,2–4,0).

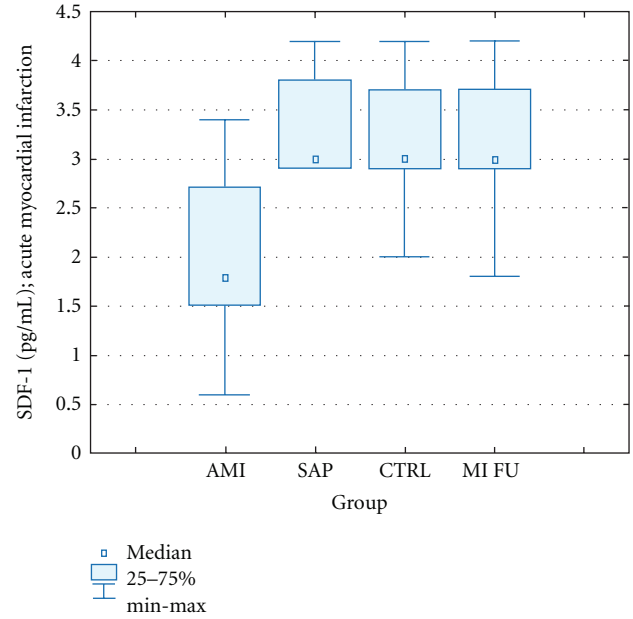

Figure 2: PlasmaSDF-1 levels. AMI: acute myocardial infarction, SAP: stable angina pectoris, CTRL; control group, MI FUL: 1-year followup. Data is presented as cell number in 1 μL of peripheral blood (median ± IQR).

consisted of ASA (all patients), statin ($n = 49$), ACEI ($n = 48$), and beta-blockers (47 patients).

3.1. Mobilization of Stem/Progenitor Cells. The absolute number of CD34+CXCR4+ cells in patients with acute MI was statistically significantly higher in comparison with SAP and CTRL groups. In 1-year followup the number of circulating CD34+CXCR4+ cells was similar in all three groups. There were no statistically significant differences in cell number between the control group and stable angina pectoris group (Figure 1).

No differences in stem cell mobilization were noted in subgroups of patients (males versus females (2,3 (0,3–8,95) versus 2,1 (0,1–8,5); $P = 0.99$], presence of type 2 diabetes [2,4 (0,1–7,6) versus 2,1 (0,1–8,9); $P = 0.31$). We found also no significant differences in patients who were on chronic treatment with statins [2,7 (0,4–7,9) versus 2,1 (0,4–7,9); $P = 0,84$], ACE-I [2,1 (0,4–7,8) versus 2,4 (0,1–8,9); $P = 0,58$].

3.2. Levels of Chemoattractants. In patients with acute MI peripheral blood SDF-1 concentration was significantly lower than in SAP patients and healthy individuals. In 1-year follow-up, there was no difference in plasma SDF-1 level among three groups. No significant differences in SDF-1 concentration between CTRL and SAP group were observed (Table 2, Figure 2).

In patients with MI G-CSF concentration was significantly higher comparing to SAP and control group. No significant differences in G-CSF concentration between CTRL and SAP group were observed. In 1-year followup, plasma G-CSF level was similar in all three groups (Table 2, Figure 3).

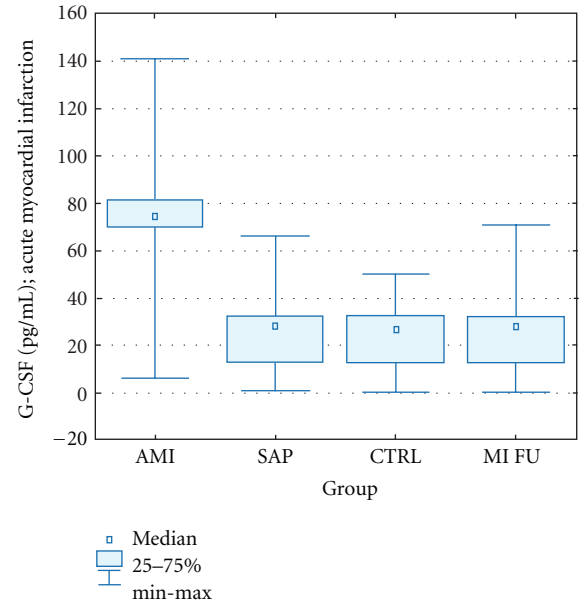

Figure 3: Plasma G-CSF levels. AMI: acute myocardial infarction, SAP: stable angina pectoris, CTRL: control group, MI FU: 1-year followup. Data is presented as cell number in 1 μL of peripheral blood (median ± IQR).

In AMI study group there was significant positive correlation between SDF-1 level and mobilized CD34+/CXCR4+ cells number ($r = 0,41$, $P = 0,023$). After 1 year, there was

Table 1: Characteristics of the study group.

	CTRL ($n = 20$)	SAP ($n = 28$)	AMI ($n = 50$)	P
Age (years) (mean ± SD)	44,6 ± 6,2	56,7 ± 11,6	58 ± 11,5	$P < 0.05$ versus CTRL
Age (years) (median ± IQR)	44 (34–54)	56 (32–75)	57 (30–79)	$P < 0.05$ versus CTRL
Male, n (%)	17 (57)	18 (60)	30 (60)	P = NS
Hypertension, n (%)	7 (23)	17 (57)	30 (60)	$P < 0.05$ versus CTRL
Hypercholesterolaemia, n (%)	8 (26)	23 (77)	35 (70)	$P < 0.05$ versus CTRL
Type 2 diabetes mellitus, n (%)	0	11 (36)	18 (36)	$P < 0.05$ versus CTRL
Smoking, n (%)	12 (40)	19 (63)	32 (64)	$P < 0.05$ versus CTRL
Family history of IHD, n (%)	8 (27)	13 (43)	24 (48)	$P < 0.05$ versus CTRL
Statins prior to hospitalization, n (%)	0	20 (67)	32 (64)	$P < 0.05$ versus CTRL
ACE inhibitors, n (%)	4 (13)	16 (53)	23 (46)	$P < 0.05$ versus CTRL
Acetylsalicylic acid, n (%)	2 (7)	28 (100)	31 (62)	$P < 0.05$ versus CTRL, SAP
Total cholesterol [mg/dL]	199 (156–256)	201 (156–256)	201,5 (122–313)	P = NS
HDL cholesterol [mg/dL]	43 (20–70)	43 (24–75)	41 (13–74)	P = NS
LDL Cholesterol [mg/dL]	97 (89–124)	100 (65–216)	105 (65–113)	P = NS
Triglycerides [mg/dL]	150 (123–200)	176,5 (84–269)	163 (76–375)	P = NS
Creatinine [mg/dL]	0,9 (0,7–1,4)	0,9 (0,8–1,3)	0,9 (0,7–14)	P = NS
Erythrocytes [$\times 10^6/\mu$L]	4,7 (4,2–5,1)	4,7 (4,3–5,1)	4,62 (4,12–5,2)	P = NS
Leucocytes [$\times 10^3/\mu$L]	6,9 (5,5–8,3)	6,6 (5,2–7,4)	10,17 ± 2,8	$P < 0.001$ versus CTRL, SAP
Monocytes [$\times 10^3/\mu$L]	0,8 (0,4–1,2)	0,7 (0,46–1,14)	0,76 (0,41–1,1)	P = NS
Platelets [$\times 10^3/\mu$L]	195 (143–246)	194 (137–251)	198 (146–250)	P = NS
Initial LVEF ≤40%, n (%)	—	—	14 (28)	
Initial CKMB [U/l]	—	—	26,5 (5–136)	
Initial TnI [ng/mL]	—	—	0,7 (0,0–18)	
Maximal CKMB [U/l]	—	—	109,5 (5–572)	
Maximal TnI [ng/mL]	—	—	4,7 (0,92–72)	
Anterior wall infarction, n (%)	—	—	30 (60)	
Multivessel CAD, n (%)	—	—	26 (52)	

Table 2

	CTRL	SAP	AMI	MI F-U (1 year)
SDF-1 [pg/mL]	3,2 (0,2–4,4)	2,9 (0,1–4,4)	1,8 (0,6–3,4)	3,0 (0,1–4,2)
P		0,77 versus CTRL	<0.0001 versus CTRL <0,0001 versus SA	0,81 versus CTRL 0,92 versus SA <0,0001 versus MI
G-CSF [pg/mL]	27 (0,7–66)	25 (0,1–50)	74 (6–141)	30 (0,8–71)
P		0,78 versus CTRL	<0,0001 versus CTRL <0,0001 versus SA	0,9 versus CTRL 0,83 versus SA <0,0001 versus MI

no significant correlation between levels of SDF-1 and G-CSF and number of circulating cells. We found no differences in SDF-1 and G-CSF levels in subgroups of patients (males versus females, presence of type 2 diabetes, and chronic treatment with statins). In patients with MI older than 50 years the number of mobilized CD34+CXCR4+ cells and plasma SDF-1 level were significantly lower than in younger patients. CD34+/CXCR4+ cells number: 2,8 (0,4–4,95) versus 5,7 (3,8–8,95); $P < 0.0001$ for patients ≥ and <50 years, respectively. SDF-1 level: 1,5 (0,6–2,4) versus 2,7 (1,4–3,4); $P = 0.004$ for patients ≥ and <50 years, respectively.

Plasma SDF-1 concentration was a single, independent prognostic factor of significant progenitor cells mobilization [Odds ratio (95% confidence interval): OR 5,8 (95% CI: 5–22); $P = 0,01$].

3.3. Left Ventricle Contractility and Remodeling. 50 patients with acute MI were evaluated by echocardiography in order to determine the correlation between circulating progenitor cell number and LVEF and remodeling (ESD, EDD). Initial echocardiographic examination revealed LVEF impairment (≤40%) in 14 individuals (28% of MI patients). Significant LVEF improvement (≥5%) during the follow-up was observed in 19 patients (38%). In 1-year observation, decreased LVEF ≤40% was diagnosed in 19 patients (38%). There were

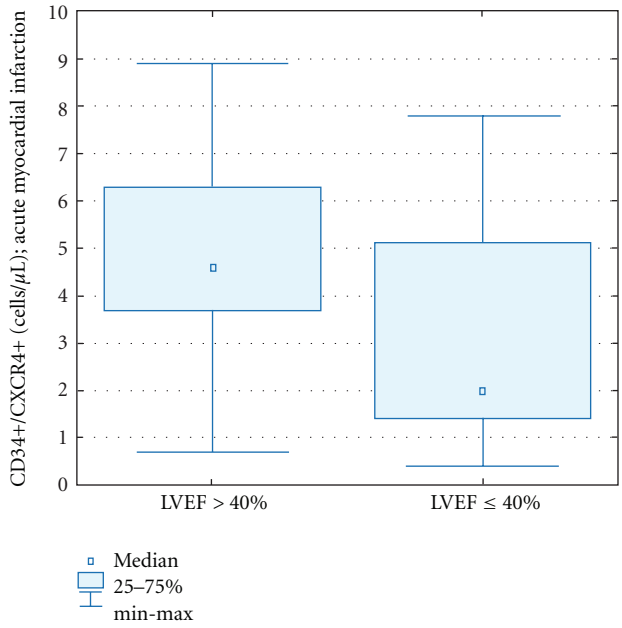

Figure 4: Circulating CD34+CXCR4+ cells in the acute phase of MI in patients with LVEF >40% versus ≤40%.

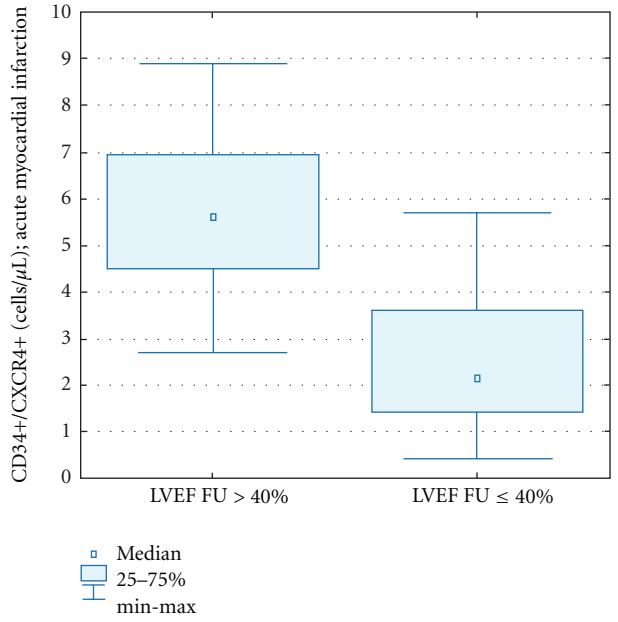

Figure 5: Mobilization of CD34+CXCR4+ cells in acute MI in patients who had preserved or reduced LVEF at 1-year followup post MI.

no significant differences between median EDD and ESD values measured 12 months after MI and during the acute phase as shown in Table 3. Also no differences in terms of medical treatment between patients with and without recovery of LVEF were found.

3.4. Mobilization of the Stem and Progenitor Cells in Relation to LVEF and Remodeling. The absolute numbers of CD34+CXCR4+ cells in acute MI was significantly lower in patients with decreased left ventricle ejection fraction (LVEF $\leq$ 40%) in the acute phase of MI [2,0 (0,4–7,8) versus 4,7 (0,7–8,9); $P = 0,028$], as well as in 1-year followup [2,3 (0,3–5,8) versus 5,5 (2,8–8,9); $P < 0.0001$] (Figures 4 and 5).

There was a significant positive correlation between CD34+CXCR4+ cells mobilization and left ventricle ejection fraction in first 24 hours after myocardial infarction ($r = 0,39$, $P = 0,03$) (Figure 6).

In control echocardiographic evaluation it was shown that CD34+CXCR4+ cells number in acute MI was positively correlated with LVEF 12 months after MI (data not shown).

There was significant positive correlation ($r = 0,41$, $P = 0,031$) between the number of mobilized progenitor cells in the acute phase of myocardial infarction and LVEF change in 12-month observation (ΔLVEF; F-U) (Figure 7).

Additionally, it was shown that in patients who had LVEF increase ≥5% in 12-month observation the number of circulating cells in the acute phase of myocardial infarction was significantly higher comparing to patients with decreased LVEF, no LVEF change and insignificant LVEF improvement [6,8 (1,9–8,9) versus 3,7 (0,4–6,8); $P < 0,0001$] (Figure 8).

Multivariate logistic regression analysis included parameters which were predictors of changes of LVEF over 1 year in

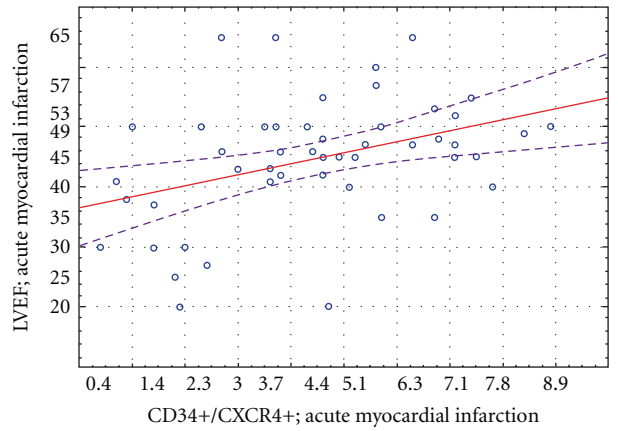

Figure 6: Correlation between CD34+CXCR4+ cells mobilization and initial LVEF value in patients with MI.

univariate model (peak values of TnI, peak activity of CK-MB, anterior localization of MI, time to reperfusion, and number of circulating CD34+CXCR4+ cells in acute MI). Only anterior localization of MI and peak values of TnI, but not number of circulating cells, were independent predictors of LVEF changes over time.

There was a significant negative correlation between mobilization of CD34+CXCR4+ cells in acute MI with ESD and EDD in the acute phase of MI as well as after 1 year (data not shown). The number of mobilized CD34+CXCR4+ cells in acute MI was inversely correlated with absolute change of ESD and EDD (Figure 9).

In MI patients with LVEF below 40% the SDF-1 levels were lower than in patients with preserved LVEF [1,45 (0,6–2,9) versus 2,0 (0,9–3,45) pg/mL; $P = 0.008$] (data

Table 3: Left ventricle ejection fraction and remodeling in 1-year follow-up in patients with MI.

Parameter	Acute MI	1 year FU	*P* value
LVEF (%)	44,7 ±10,2	45,9 ± 10,5	0,17
EDD (mm)	51,6 ± 5,5	51,1 ± 5,5	0,9
ESD (mm)	33,4,6 ± 3,9	35,1 ± 5,1	0,08
ΔLVEF (%)	—	1,2 ± 6,7	—
ΔESD (mm)	—	2,5 ± 3,3	—
ΔEDD (mm)	—	0,5 ± 2,9	—

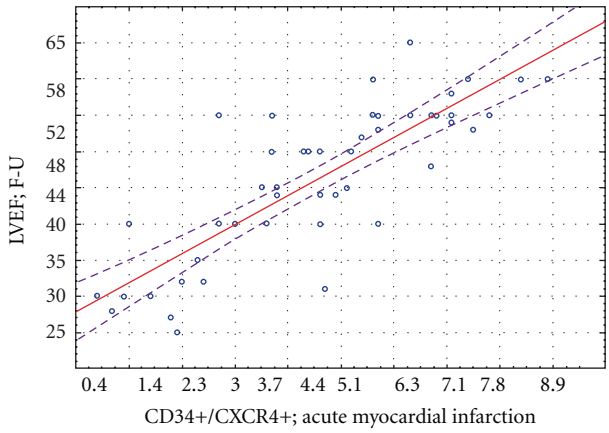

Figure 7: Correlation between peripheral blood CD34+/CXCR4+ progenitor cells number and the absolute LVEF change 12 months post MI.

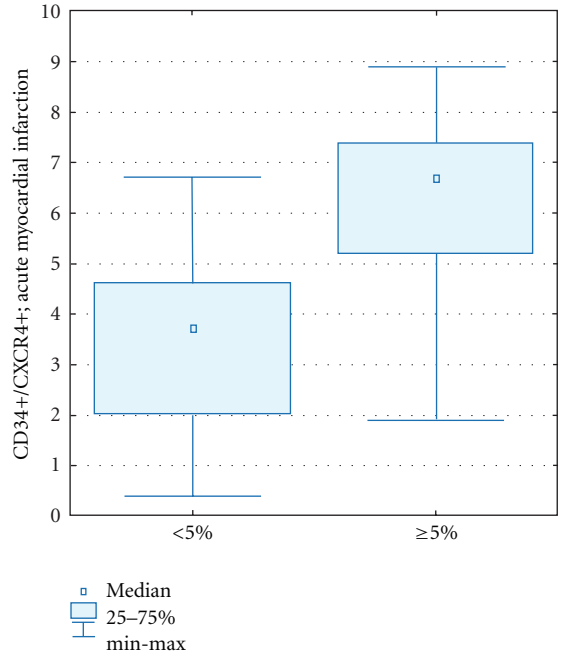

Figure 8: Comparison of CD34+/CXCR4+ progenitor cells mobilization in the acute phase of myocardial infarction in patients with the absolute LVEF improvement ≥5% (Δ ≥ 5%); 12-month clinical followup.

not shown). There were no significant correlations between chemoattractants and LVEF and LV remodeling after 1 year. Mobilization of CD34+CXCR4+ cells measured in acute MI was negatively correlated with maximum TnI levels (Figure 10).

NT-proBNP levels in patients with acute MI were significantly higher than in SA group [170 (34–860) versus 80 (23–167) pg/mL; $P < 0,0001$]. Number of circulating cells in acute MI was significantly negatively correlated with plasma NT-proBNP levels ($r = -0,48$, $P = 0.03$). In patients with MI, high sensitive C-reactive protein (hsCRP) level was statistically significantly increased. In 1 year follow-up, the hsCRP level were similar in all three groups (Figure 11).

There were no significant correlations between hsCRP levels and mobilization of CD34+CXCR4+ in acute MI ($r = -0.17$, $P = 0.22$).

3.5. Ergospirometry and Functional Status. The ergospirometry test was carried out in 48 of 50 patients after 1 year after MI. Results are shown in Table 4.

Only variable that correlated with mobilization of CD34+CXCR4+ cells was VO_2 peak ($r = 0.34$, $P = 0.01$). Similarly, there were no differences in number of circulating cells and chemoattractants in patients stratified according to NYHA class at 1-year followup.

4. Discussion

Acute MI triggers the release into peripheral blood of BM-derived stem and progenitor cells, such as EPCs, VSELs, HSCs, and MSCs. In present study we provided evidence that mobilization of CD34+CXCR4+ cells in acute MI was significantly correlated with improvement of LVEF and LV remodeling in 1-year followup. Reduced mobilization of CD34+CXCR4+ cells in acute phase of MI was associated with more significant impairment of LVEF and greater infarct size measured as the release of TnI. We evaluated the mobilization of CD34+CXCR4+ cells in acute MI in comparison to patients with stable angina and control group without CAD. According to our previously published results the maximum mobilization of cells occurred early within 12 hours after the onset of ischemia and the number of circulating cells increased approximately 2.3-fold. The number of these circulating cells after 1 year was comparable to patients with stable CAD and healthy subjects. Mobilization of the stem and progenitor cells might therefore be considered a part of an inflammatory response in response to myocardial

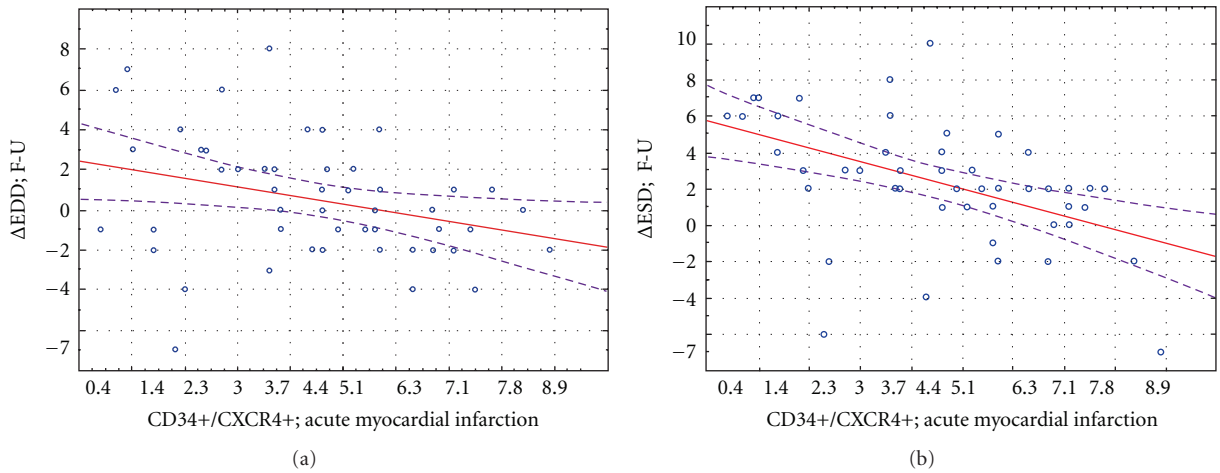

Figure 9: Correlation of CD34+CXCR4+ progenitor cell number in the acute phase of MI with the absolute EDD change (ΔEDD) and ESD (ΔESD) in 12-month clinical observation.

Table 4: Results of ergospirometry.

	VO_2 peak	VE/VCO_2 slope	VE/VCO_2 peak/rest	VE/VCO_2 peak	VE/VCO_2 rest
Median (Range)	26.0 (13–33)	29.7 (18,1–41,6)	0.88 (0,68–0,99)	33.13 (24–50)	42.98 (30–51)

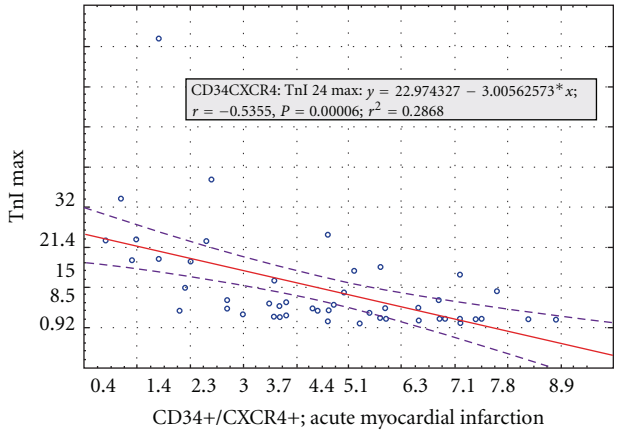

Figure 10: Correlation between CD34+CXCR4+ cells mobilization in the acute phase of MI and maximum levels of troponin I.

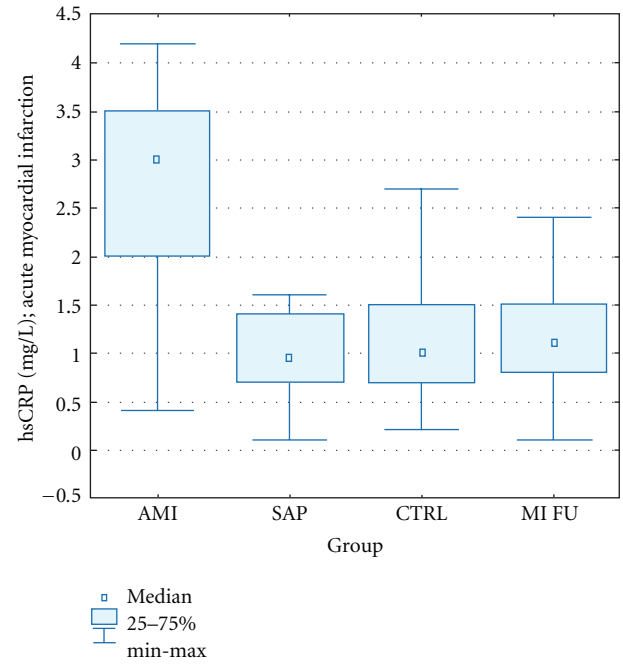

Figure 11: Plasma hsCRP levels. Patients: with acute myocardial infarction: AMI, with stable angina pectoris: SAP, control group: CTRL, 1-year followup: MI FU. $P = 0,47$ SAP versus CTRL; $P < 0,0001$ AMI versus CTRL; $P < 0,0001$ AMI versus SAP; $P = 0,21$ MI F-U versus CTRL; $P = 0,73$ MI FU versus SAP; $P < 0,0001$ MI F-U versus AMI.

ischemia and necrosis. Current observations are consistent with other studies investigating mobilization of EPCs and HSCs which showed rapid release of cells. Therefore measurement of CD34+CXCR4+ cells at admission reflects in our opinion the maximum mobilization triggered by acute MI [3–5].

Our previous studies demonstrated that CD34+CXCR4+ and c-met+ cells are present in increased numbers for first 2-3 days and is gradually reduced within a week [4]. Accordingly Leone et al. showed that in acute MI several populations of cells CD34+CD33+, CD34+CD38+, CD34+CD117+, and CD34+VEGFR2+ are mobilized within 6 hours after the onset of ischemia and returned to levels comparable with stable CAD within 2 months [17]. Leone et al. also confirmed the rapid release of cells within 24 hours after MI [17]. Conversely, Shintani et al. showed that the peak number of CD34+ cells occurred later, about 7 days after the onset of ischemia [3]. Mobilization of BM cells was

confirmed also in non-ST-segment elevation acute coronary syndromes [13].

So far there was no prospective study which investigated the relations between mobilization of BM cells and recovery of LV contractility following acute MI. In the present study we showed that the mobilization of CD34+CXCR4+ cells is positively correlated with LVEF both measured in the acute phase as well as its recovery over 1-year followup. We also showed that almost 30% of patients had reduced LVEF $\leq$40% and these patients had significantly less circulating cells than patients with preserved LVEF. Reduced mobilization was also observed in patients with no significant improvement of LVEF following reperfusion over the long-term followup. Patients with a significant increase of LVEF defined as increase $\geq$5% had significantly higher number of circulating cells. In addition our study suggests that the release of CD34+CXCR4+ cells is inversely correlated with LV remodeling measured by absolute increase of EDD and ESD over 1 year. Overall the findings show consistently that in patients with reduced LVEF and lack of significant improvement of contractility as well as more significant remodeling the mobilization of CD34+CXCR4+ cells in acute MI is reduced.

There is a paucity of data on such associations in the literature. Leone et al. assessed the correlation of CD34+ with LVEF in 54 patients with acute MI. Number of cells was measured after 1 year. Authors showed that mobilization of CD34+ cells was an independent predictor of improvement of LVEF and it was positively correlated with absolute increase of LVEF and negatively with wall motion score index (WMSI) and end-systolic volume. Patients with most significant improvement of LVEF as well as a reduction of WMSI and LV volumes had also higher number of circulating CD34+ cells 1 year after MI [16]. In this study however only 16 patients were treated with primary PCI and 12 had no reperfusion treatment at all. Also only 45% of cells were CXCR4+, so this study evaluated different population of BM cells [17]. Conversely Massa et al. showed no correlation between EPCs, HSCs, and LVEF in patients with acute MI [18]. In the present study we enrolled only patients treated with primary PCI and final TIMI3 flow to reduce the bias caused by inclusion of patients without proper reperfusion which translates into LV remodeling. We did not find any differences in mobilization of cells in subgroups of patients stratified by sex, presence of diabetes, hypertension, and obesity. Interestingly we observed that the mobilization was lower in older patients in comparison to those younger than 50 years. Proper interpretation of the results requires the consideration of other factors that can modulate the process of mobilization, including age, medications, and profile of CAD risk factors. Generally the mobilization and function of circulating SPC is reduced in elderly and diabetes. On the other hand statins improve the mobilization, viability, and function of these cells; however most available data referred to EPCs. Our population of cells was distinct from EPCs and definitely more heterogenous. On the other hand subpopulation of CXCR4+ cells (CD133+CXCR4+ VSELs) is indeed reduced in diabetic patients with acute MI [5, 13].

The presence of correlations between LV contractility, remodeling, and mobilization of cells which theoretically might contribute to tissue repair is clearly not a proof of a causal relationship but only a hypothesis-generating concept. We hypothesized that some populations of BM cells might be particularly intriguing because of their ability to express early cardiac and endothelial lineage markers which suggest they might play a role in cardiac reparatory reaction following MI. We previously showed that some populations of circulating cells (CD34+CXR4+, VSELS) express mRNA for early cardiac (Nkx2.5/Csx, GATA-4) muscle and endothelial markers (VE-cadherin, von Willebrand factor). The increased expression of these markers is in temporal correlation with maximum mobilization of CXCR4+ cells [4]. We also demonstrated that acute MI triggers mobilization of VSELs expressing early developmental markers (Oct-4, Nanog, SSEA-4). On the other hand, murine VSELS showed potential for differentiation into cardiac myocytes [6]. It seems that mobilization of CXCR4+ cells reflects not only an inflammatory reaction following MI, but is also a part of repair mechanism. Interestingly the potential for differentiation of circulating progenitor cells was shown to be correlated with improvement of LVEF after MI as shown by Numaguchi et al. [18]. In our study the levels of chemoattractants (SDF-1, G-CSF) were not significantly correlated with improvement of LVEF or remodeling. Release of TnI in acute MI is strongly correlated with the infarction size and is a predictor of LVEF recovery following MI. Other important predictors are anterior localization and time from the onset of symptoms to reperfusion [16]. In our hands the mobilization of CD34+CXCR4+ cells was inversely correlated to maximum levels of TnI and activity of CK-MB, which suggests that patients with blunted mobilization of stem cells developed larger infarcts. This might in part explain reduced LVEF recovery and increased remodeling in this group. Massa as well as Leone et al. however found no association between circulating cells and myocardial necrosis markers [5, 16, 17]. In addition Voo et al. showed that number of EPCs is positively correlated with myocardial necrosis and levels of CRP [19]. In multivariate analysis however the number of CD34+CXCR4+ cells was not an independent predictor of LVEF recovery.

We investigated if the mobilization of BM cells is related to functional status of the patients in 1-year follow-up. We showed no differences between patients presenting with MI with or without heart failure (Killip-Kimball class). In the long-term follow-up we used cardiopulmonary exercise test which is a noninvasive, reproducible, and reliable tool for evaluation of exercise tolerance. Such study was completed by 96% of patients. The only parameter which showed a positive correlation with the number of circulating cells measured 1 year after MI was VO_2 peak. It seems that improved LVEF and less remodeling in patients with a higher number of circulating cells might have translated into better exercise capacity. NT-proBNP is a significant prognostic marker in acute coronary syndromes and heart failure [21]. We found increased levels of NT-proBNP in patients with MI which were inversely correlated with LVEF, but also with the number of circulating CD34+CXCR4+ cells. Valgimigli et al.

showed that number of circulating CD34+CD45+ cells and EPCs in patients with chronic heart failure is reduced in higher NYHA class, inversely correlated with BNP and positively with peak VO_2 in ergospirometry [21]. We found no evidence of correlation between cell mobilization, chemoattractants levels with NYHA and CCS class in 1-year followup. Several other studies showed that in patients with heart failure the numbers of circulating stem and progenitor cells as well as their functional capacity are reduced [15, 22].

Mobilization and homing of circulating stem and progenitor cells is regulated by expression of chemoattractants, such as chemokines (SDF-1), growth factors (VEGF) or cytokines (G-CSF) as well as activation of the complement cascade and bioactive phospholipids.

Several chemoattractants, such as SDF-1, LIF, and HGF, are expressed in the myocardium in particular in infarct border-zone. This suggests that cells with receptors for chemoattractant are preferentially taken up in these areas [22, 23]. Our study showed increased levels of G-CSF and decrease in SDF-1 levels in acute MI. Previously we demonstrated that following MI the baseline low levels of SDF-1 increase over time, which corresponds with peak expression of SDF-1 in the myocardium which occurred later than mobilization of CXCR4+ cells [7]. SDF-1 levels are predictors of significant mobilization of CD34+ cells [4]. Probably for stem cell homing the local expression of SDF-1 in the heart is more important than blood levels which show high individual variability. We found however no significant correlations between plasma G-CSF levels and cell mobilization as well as any of the clinical parameters. Similarly there were no significant correlations between increased levels of hsCRP in patients with acute MI and cell mobilization.

The limitations of the study are relatively small sample size and use of echocardiography instead of MRI in particular in the analysis of comparison between patients with and without significant (>5%) improvement of LVEF. Additionally, we compared the release of SPC in acute MI to number of cells in patients with stable CAD and control subjects without CAD. Latter two groups were different in regard to age and use of medications from patients with MI, so we could not have excluded the bias, because both factors modulate the number of circulating cells.

4.1. Summary. Mobilization of CD34+CXCR4+ cells in acute MI shows significant positive correlation with left ventricular ejection fraction and inverse correlation with infarct size and NT-proBNP levels. Number of circulating cells is lower in patients with reduced LVEF, LV remodeling and those without significant improvement of LV contractility in 1-year follow-up.

Authors' Contribution

The first two authors contributed equally to this work.

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Research Article

Differential Expression of Sphingosine-1-Phosphate Receptors in Abdominal Aortic Aneurysms

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Objective. Inflammation plays a key role in the pathophysiology of abdominal aortic aneurysms (AAAs). Newly discovered Sphingosine-1-Phosphate Receptors (S1P receptors) are critical in modulating inflammatory response via prostaglandin production. The aim of the current study was to investigate the expression of different S1P receptors in AAAs and compared with normal aortas at the protein level. *Materials and Methods*. Aortic specimens were harvested during aortic reconstructive surgery for the AAA group or during organ transplant for the control group. The protein expression of S1P1, 2 and 3 in AAAs and normal aortas was assessed by Western blotting and immunohistochemical analysis. *Results*. There were 40 AAAs and 20 control aortas collected for the receptor analysis. For Western blot analysis, S1P1 expression was not detected in either group; S1P2 protein was constitutively detected in both types of aortas but its expression level was significantly decreased by 73% ($P < 0.05$) in AAAs compared with the control group. In contrast, strong S1P3 expression was detected in AAAs aortas but not in normal aortas. Immumohistochemical staining showed similar results, except a weak S1P3 signal was detectable in normal aortas. *Conclusions*. Western blot and staining results consistently showed the down-regulation of the S1P2 protein with simultaneous up-regulation of the S1P3 protein in AAAs. Since those newly discovered receptors play an important role in the inflammatory cascade, the modulating of S1P signaling, particularly via S1P2 and S1P3, could represent novel therapeutic targets in future AAA treatments.

1. Introduction

Abdominal aortic aneurysm (AAA) is the localized dilation of the infrarenal aorta. If surgical treatment is not applicable, an AAA progresses to rupture with a high mortality rate and causes 1%–3% of elderly male deaths in developed countries each year [1]. Analysis of the aneurysmal wall has demonstrated that connective tissue degradation, increased atherosclerosis, and chronic inflammation are the common pathological features of AAAs [1–3].

Sphingosine-1-phosphate (S1P) is a newly discovered low-molecular-weight zwitterionic lysophospholipid molecule that is generated from the metabolism of sphingomyelin by a series of enzymes including sphingosine kinase, S1P phosphatase, and S1P lyase in mammals [4]. The main sources of S1P are platelet cells in plasma, while other cell types such as erythrocytes, neutrophils, and mononuclear cells can also produce and release S1P upon activation [4, 5]. S1P exerts a wide range of physiological activities, particularly inflammatory reactions through the interactions with five different receptor subtypes 1, 2, 3, 4, and 5. They are the members of the endothelial differentiation gene family of G protein-coupled receptors [4], and differential expressions of S1P receptors are thought to modulate the cellular inflammatory response [6]. A precise S1P/S1P receptor balance is found to be responsible for the signaling of cell growth and regulation of cell metabolism in mammal [7, 8]. An imbalance of this system also participates in pathologic conditions such as cancer and inflammatory diseases [9, 10]. S1P2 is the major expressed S1P receptor, while S1P1 or S1P3 is only weakly expressed in healthy vascular endothelial cells [11] and vascular smooth muscle cells (VSMCs) [12]. Consistently with this receptor multiplicity and pleiotropic signaling mechanisms, S1P receptors influence numerous cell functions. Particularly, differential expressions of S1P receptors have been demonstrated to either promote or inhibit the inflammatory infiltration in diverse cell types by inducing cyclooxygenase 2 (COX-2) expression [6]

with subsequent prostaglandin E_2 (PGE_2) or prostacyclin (PGI_2) production [13–15]. Our previous study showed that inflammatory mediators such as Cox-2 and prostaglandin E_2 are also widely expressed in AAA explants [16]. Those phenomena implicating S1P receptors may play some roles in the pathogenesis of AAA.

S1P1, S1P2, and S1P3 receptors are the major S1P receptor subtypes in the vascular system [11, 12]. However, the expressions of these three S1P receptors in AAA remain unknown. In the present study, we aimed to investigate the S1P1, 2 and 3 receptor protein expressions in AAAs and compare them with healthy aortas.

2. Materials and Methods

2.1. Human Abdominal Aorta Tissues Collection. Cross-sections of aneurysm wall, which were dissected of luminal thrombus, were removed from the AAA patients who underwent open surgical aneurysmal repair in a local hospital. Control aortic tissues were obtained from the corresponding location of healthy organ donors without known cardiovascular diseases and connective tissue disorders during the transplant operation. Aneurysmal patients with the Marfan syndrome and other connective tissue disorders were excluded from this study.

Each collected specimen was thoroughly washed with normal saline solution, and then the tunica intima elimination procedure was conducted using a scalpel and forceps. All specimens were divided into two parts for the western blotting which was kept in a -80°C freezer and immunohistochemical analysis, respectively.

All experiments were performed with the approval from the local institution's ethics committee. Informed consent was obtained from AAA patients and organ donors' relatives.

2.2. Western Blot Analysis. Frozen tissues were first thawed and then lysed with cell lysis buffer (Cell Signaling. Technology, Danvers, MA, USA) containing protease inhibitor (Roche, Basel, Switzerland). The protein concentration of each specimen was measured based on the Bradford method utilizing the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA) with bovine serum albumin (BSA) as the standard. After the protein denaturing procedure with loading buffer (pH 6.8 24 mM Tris-HCl, 684 mM glycerol, 14 mM SDS, 142 mM beta-mercaptoethanol, 0.3 mM bromophenol blue), each sample (50 μg) was resolved on 12% SDS-polyacrylamide gel electrophoresis (PAGE) gel (Bio-Rad Laboratories) at room temperature then transferred onto a polyvinylidene fluoride (PVDF) membrane (Bio-Rad Laboratories) at 4°C. After blocking in 10% TBS–0.01% Tween 20 (TBST) diluting nonfatty milk (Bio-Rad Laboratories) for two hours at room temperature, the membranes were then incubated overnight at 4°C with primary antibodies against S1P1 receptor (Catalogue no. sc-48356, dilution 1 : 100, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or S1P2 receptor (Catalogue no. sc-25491, dilution 1 : 200, Santa Cruz Biotechnology) or S1P3 receptor (Catalogue no. sc-30024, dilution 1 : 100, Santa Cruz Biotechnology) with GAPDH (1 : 1000, Cell Signaling Technology) as the positive control. After membrane washing using TBST solution, HRP-conjugated goat anti-mouse (1 : 2000) or goat anti-rabbit (1 : 4000) secondary antibody (Dako, Glostrup, Denmark) was added and the membranes were then incubated for 1 hour at room temperature. After washing, signals were visualized by luminol reagents (Bio-Rad Laboratories) and the densitometry of each exposing blotting was analyzed by ImageJ 1.44 software (National Institutes of Health, Bethesda, MD, USA). The relative expression of the studied receptors' protein was calculated by the detected signal divided by the internal positive control (GAPDH) expression signal in each sample.

2.3. Immunohistochemical Study. The staining procedure was performed on paraffin-embedded aortic tissue (5 μm) sections according to the manufacturer's instructions (Dako-Cytomation EnVision + System-HRP (DAB) Kit (Dako)). Specificity of S1P receptor antibodies was firstly validated by positive and negative tests using healthy adult rat brain paraffin sections. Briefly, all sections were antigen retrieved with boiling sodium citrate buffer (pH 6) and incubated with either mouse anti-S1P1 receptor antibody (1 : 25), rabbit anti-S1P2 receptor antibody (1 : 100), or rabbit anti-S1P3 receptor antibody (1 : 100) (Santa Cruz Biotechnology) overnight at 4°C. After staining, all specimens were subjected to the dehydration procedure and sealed for microscopy observation.

To avoid staining underestimation due to considerable regional variations, 5 continuous × 200 microscopy views of each stained specimen, which had the largest amount of positive stained VSMCs, were captured and recorded (Nikon, Tokyo, Japan). Two researchers scored the positive immunostaining using the scoring system according to the Wang and colleagues study [17]. Briefly, a proportion subscore from 0 to 4 (i.e., 0—0% positive stained, 1—1%–25% positive stained, 2—26%–50% positive stained, 3—51%–75% positive stained, 4—76%–100% positive stained) and an intensity subscore from 0 to 3 (i.e., 0: no staining, 1: weak staining, 2: moderate staining, 3: intense staining) were first assigned by each observer for each slide. A weighted score was then determined by multiplying the proportion subscore and the intensity subscore. Finally, a mean value of the five weighted scores for each specimen was calculated.

2.4. Statistical Analysis. All data were expressed as means ± SD. Statistical analysis was performed by SPSS 18.0 software (SPSS, Chicago, IL, USA). Any statistical differences between the two groups' experimental results were determined by independent sample t-tests. Correction for ages and sex of patients on receptor expression levels were made using a linear model. A P value < 0.05 was considered as statistically significant.

3. Results

3.1. Patient Characteristics. There were 40 AAA specimens and 20 control aortas obtained from the corresponding

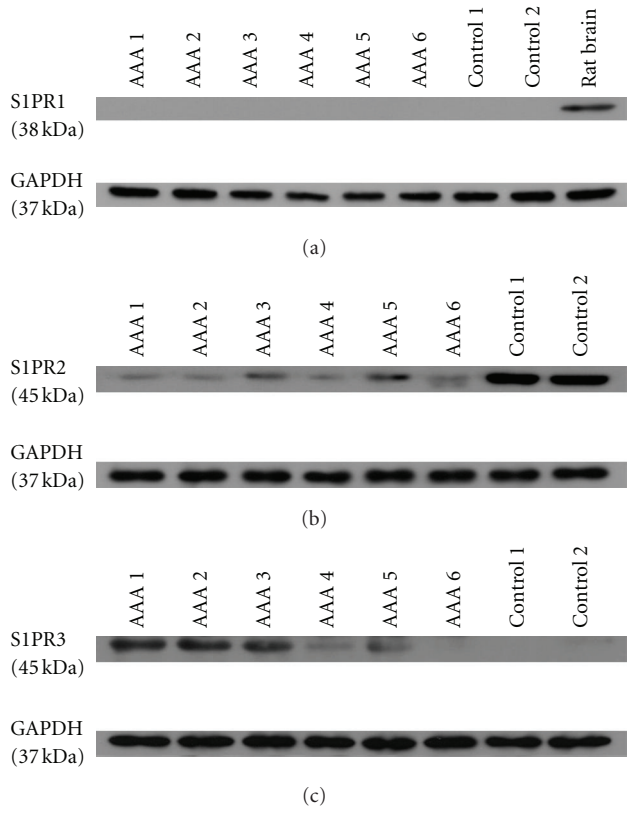


Figure 1: Representative pictures of western blot analysis of S1P receptors in AAA and control aortic tissues. S1P1 expression was undetectable in both AAA and normal aortas, while it became detectable in rat brain tissues (a). Expression level of S1P2 protein was significantly decreased in AAAs compared with control aortas (b). S1P3 expression was detectable in AAAs only (c). GAPDH antibody was used as internal positive control in each WB experiment.

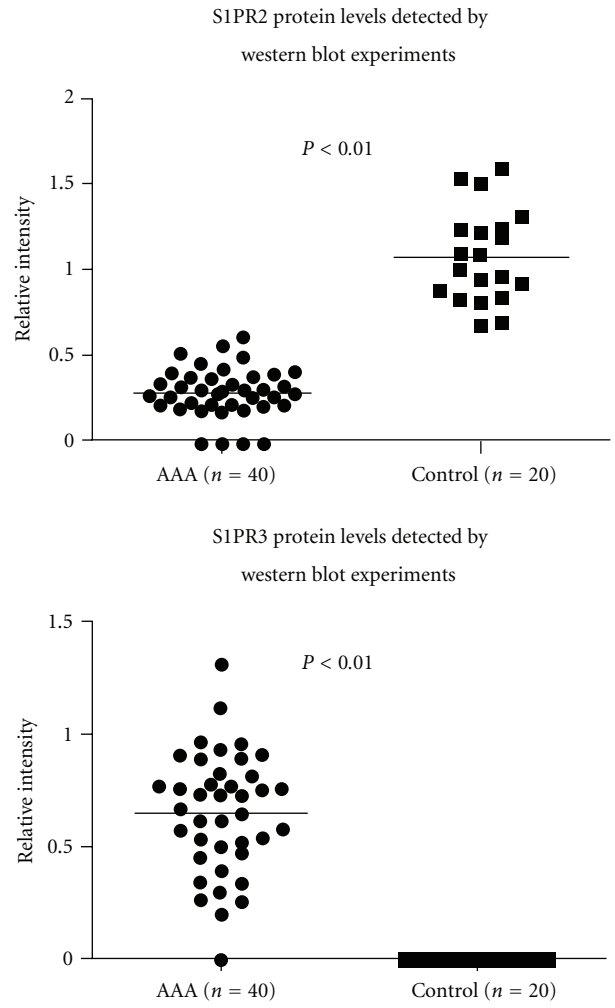


Figure 2: Quantitative analysis of S1P2 (upper panel) and 3 (lower panel) protein expression levels in western blot analysis. The relative S1P2 protein level was decreased by 73% ($P < 0.05$) in AAAs (relative expression intensity of 0.29) compared with normal aortas (relative expression intensity of 1.08). S1PR3 protein levels were significantly upregulated in AAA tissues with average relative intensity of 0.65, whereas it was undetectable in normal aortas.

surgical patients and organ donors. Most of patients were male, and the control patients were younger than the AAA patients. Patient characteristics are listed in Table 1.

3.2. Western Blot Analysis. S1P1 receptor protein (38 kDa) was undetectable in both tissues (Figure 1(a)). For S1P2 receptor protein (45 kDa), positive signals were detected in both AAA and control aortic tissues, with AAA tissues had a significantly lower protein expression level compared with control aortas (Figure 1(b)). In contrast, S1P3 receptor protein (45 kDa) was highly expressed in AAA aortas but was undetectable in control aortas (Figure 1(c)).

The relative intensities of S1P1 and 2 receptors expression by western blot analysis are shown in Figure 2 upper and lower panels, respectively. The protein level of S1P2 receptor was decreased by 73% ($P < 0.05$) in the AAA tissues (mean relative intensity of 0.29) compared with the control aortic tissues (mean relative intensity of 1.08). S1PR3 protein levels were significantly upregulated in AAA tissues with average relative intensity of 0.65, whereas it was undetectable in normal aortas.

3.3. Immunohistochemical Staining Analysis. S1P1 receptor expression level was undetectable in both AAA (a) and normal aortas (b), as shown in Figure 3. A Positive control of S1P1 receptor was performed in healthy adult rat brain for validating its specificity (Figure 3(c)). S1P2 receptor protein was expressed in both types of aortas, with pronounced S1P2 receptor staining observed in control aortas (Figure 4). S1P3 receptor protein was found in the AAA tissues (Figure 5(a)), but it was almost undetectable in normal aortas (Figure 5(b)). The staining scores of both types of tissues sections are shown in Table 2.

Positive staining of S1P2 and S1P3 receptors in the aortic walls showed that they were localized in VSMC plasma membrane and cytoplasm but absent in the nucleus (Figures 4 and 5).

Table 1: Patient characteristics of AAA patients group and control group.

	AAA group ($n = 40$)	Control group ($n = 20$)
Age (mean age ± SD)	70.08 ± 9.72 yrs	42.05 ± 13.55 yrs
Gender (male %)	95%	40%
Smoking (%)	67.5%	—
Hypertension (%)	65%	5%
Diabetes (%)	7.5%	0%
Cardiac disease (%)	45%	0%
Renal disease (%)	27.5%	5%

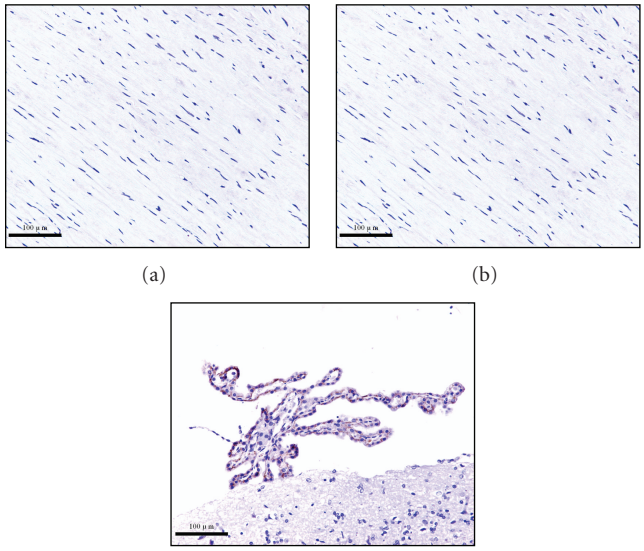


Figure 3: Representative staining pictures of S1P1 protein in AAA (a), control aortic (b) sections, and healthy adult rat brain paraffin sections (c) (×200). S1P1 protein was undetectable in both types of tissues.

Table 2: Staining scores of immunohistochemical analysis of S1P receptors in both types of tissues.

	AAA group ($n = 40$)	Control group ($n = 20$)
	Mean staining score (range)	
S1P1 receptor	—	—
S1P2 receptor	2.4 (0.6–4.8)	8.3 (5.2–10.8)
S1P3 receptor	5.2 (0.6–10.6)	0.25 (0–0.8)

3.4. Correction for Ages and Sex. Giving the age and sex discrepancy existed in the patients, corrections for ages and sex of the receptor protein expressions was made. The association between S1P2 and S1P3 protein (both IHC and WB expression levels) in the patients were robust to correction in a linear model with age and sex ($P < 0.008$).

4. Discussion

Among the studied S1P receptors, only S1P2 and S1P3 receptor proteins were differentially expressed in AAA tissues compared with the control aortas, while S1P1 receptor protein was absent in both types of tissues. Differential S1P receptors expressions have been shown to participate in diverse physiological processes, such as cell survival and apoptosis [18], and pathological processes, such as angiogenesis, inflammation, cancerogenesis, and immune regulation [9]. Inflammation is one of the common pathological features of AAAs [3]. Thus, the present novel findings may implicate the importance of these receptors in

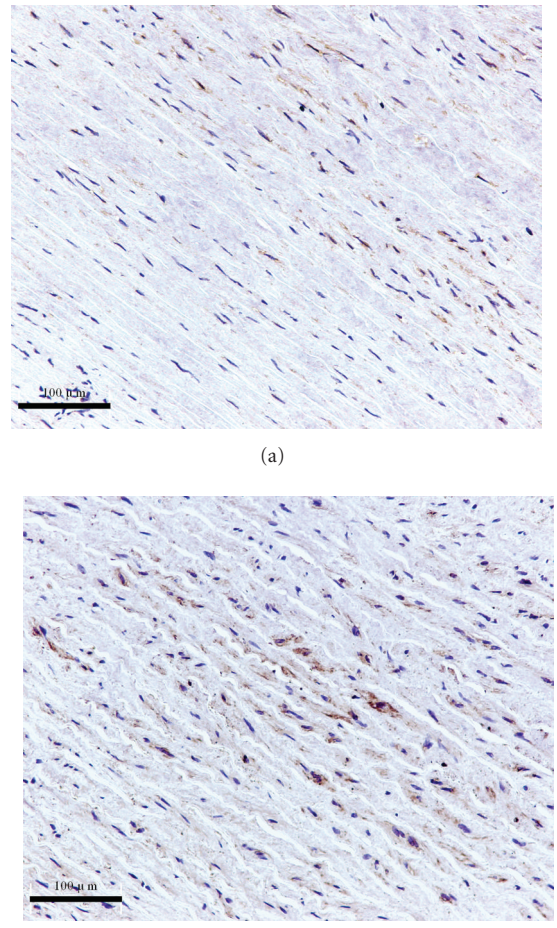
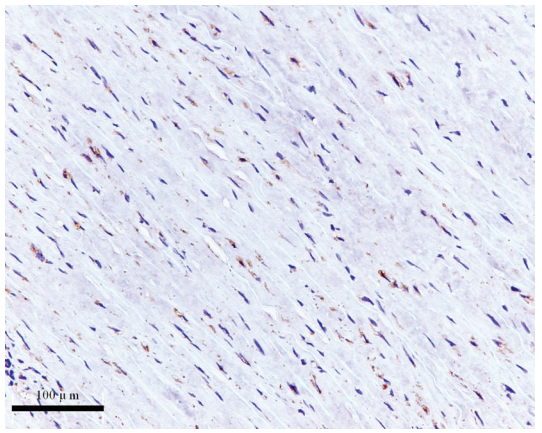
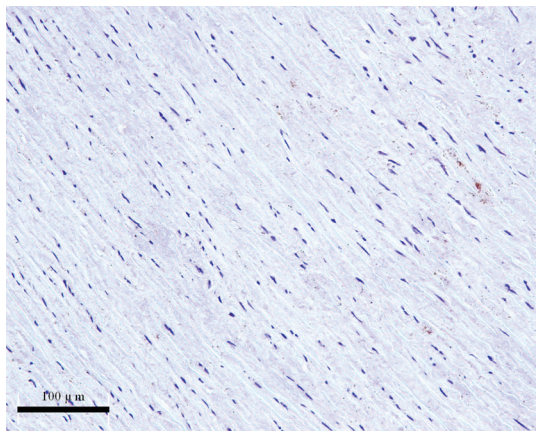


Figure 4: Representative staining pictures of S1P2 in AAAs and control aortic sections (×200). S1P2 protein was more pronounced in the normal aortas (b) than that in the AAAs (a).



(a)



(b)

Figure 5: Representative staining pictures of S1P3 in AAA and control aortic sections (×200). More S1P3 protein was found in the AAA (a) than that in the normal aortas, in which it was almost undetectable.

the inflammation attribute to AAA pathogenesis. Nevertheless, atherosclerosis is indeed regarded as a chronic inflammatory disease with atherosclerotic plaques containing inflammatory infiltrates, which implicated in the formation of AAA [19]. Thus, the possibility of S1P receptor expression related to the atherosclerotic event cannot be excluded.

S1P1 receptor is undetectable in both types of aortic tissues. Other researchers found that only some specific cell types, such as endothelial cells, cardiomyocytes, neural stem cells, as well as B cells, and T cells, express marked S1P1 [4]. Healthy rat adults' VSMCs express S1P1 receptor weakly [20], and the deletion of S1P1 receptor is embryonically lethal since it causes the failure of dorsal migration of VSMCs to form the tunica media layer of arteries [21], suggesting that S1P1 receptor should be critical in vascular development rather than in maintaining VSMCs metabolism [21]. A more recent study suggested that S1P1 is involved in the phenotype regulation of adult smooth muscle cells [22]. They utilized a rat carotid artery balloon injury model and demonstrated that there was a transient over expression and activation of S1P1 receptor after injury. This action can facilitate VSMCs transfer into the proliferative and migratory phenotype. However, such S1P1 receptor over expression will be restored to the basal value by 7 days after injury, suggesting that this irritable S1P1 receptor activation may be a short-term injury response. Thus, we postulated that the S1P1 receptor protein may be transiently increased during early AAA lesion, but its high expression will subsequently return to the basal level or become undetectable at the advanced stage of AAA development. In addition, S1P1 receptor is possibly responsible for the development of circulation system and expressed in the endothelial cells, rather than expressed in smooth muscle cells in mature aortas [21] which may explain for absent of S1P1 levels in the late stages of aneurysm.

S1P2 receptor protein was detected in both types of aortic tissues, particularly in control aortas. Indeed, S1P2 receptor has been previously shown to be the major S1P receptor expressing population in a wide variety of tissues in humans, like vascular endothelial [11] and smooth muscle cells [12], but not in inflammatory infiltrates [9]. This particular receptor can facilitate VSMCs' contractile phenotype expressions and negatively regulate their proliferation and migration [22]. Moreover, S1P2 receptor is capable of inducing Cox-2 expression and producing prostacyclin (PGI_2) in response to exogenous S1P stimulus [15, 23, 24]. PGI_2 possesses anti-inflammatory functions and simultaneously relaxes VSMCs and suppresses their proliferation and migration [25]. Thus, the decreased expression of anti-inflammatory S1P2 receptor in VSMCs of AAAs, and probably not expressed in the inflammatory infiltrates, may impair the production of PGI_2 and ultimately lead to the pronounced inflammation response in AAA patients [16]. Therefore, the S1P2 receptor downregulation of VSMCs is obviously an important etiological factor in AAA development.

Markedly S1P3 receptor protein was found in AAA tissues. S1P3 receptor possesses a promoting inflammatory response property as it can induce Cox-2 expression and concomitant PGE_2 production in various cell types [13, 14, 26, 27]. As a pronounced inflammatory infiltrate, PGE_2 was also found in AAA explants in our laboratory previously [28] though the extent of its involvement in vascular inflammation is still unclear. In addition, a very recent study suggested that S1P3 mediates the chemotactic effect of its ligand-S1P in macrophages *in vitro* and *in vivo*, which plays a crucial role in atherosclerosis by promoting inflammatory monocyte/macrophage recruitment and altering smooth muscle cell behavior [29]. We suggested that the S1P3 receptor protein may be critical in the strengthened inflammatory response and thus atheroslcerosis via the chemotactic property and the PGE_2 pathway during AAA development. However, the causal relationship between the inflammatory cells recruitment and the prostaglandins needed to be further explored.

In the present study, a weak S1P3 staining signal was found in normal aortas, though it became undetectable using western blot analysis. This staining result was consistent with Ryu et al. study that S1P3 is found to be weakly expressed in human healthy VSMCs [12].

Though age and sex discrepancy existed in the studied patients, the present analysis found that the expression of

the S1P2 and 3 receptor proteins are not age and sex dependent. Thus, the up- or downregulated receptor probably related to the inflammatory cascade underlying the AAA pathogenesis but is not simply a feature of aging or sex difference.

The limitation of the present study is only an observational analysis on surgical aneurysmal aortas. Thus, we cannot extrapolate the observations to the initiation or promotion of aneurysm formation. Nevertheless, the present work has provided evidence that aneurysmal aortic tissue exhibits a decreased activity of S1P2 and enhanced S1P3 receptor, which may contribute to the inflammation of aortic walls involved in AAA pathology.

Our findings of the differential expression of S1P receptors in AAA compared with normal aortas are novel and may be helpful to delineate the important inflammatory mechanisms in AAA development. This investigation has provided a new concept in the inflammatory response in the lesions, and the regulation of S1P receptor via S1P2 and S1P3 may open a new regime for AAA treatment in the future.

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Review Article

C-Peptide: A New Mediator of Atherosclerosis in Diabetes

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Diabetes type 2 and insulin resistance are the risk factors for cardiovascular disease. It is already known that atherosclerosis is an inflammatory disease, and a lot of different factors are involved in its onset. C-peptide is a cleavage product of proinsulin, an active substance with a number of effects within different complications of diabetes. In this paper we discuss the role of C-peptide and its effects in the development of atherosclerosis in type 2 diabetic patients.

1. Introduction

C-peptide is a small 31-amino acid peptide, and it is cleaved from proinsulin in the synthesis of insulin [1]. Proinsulin consists of A and B chain and connecting peptide in the middle, called C-peptide. Cleavage of proinsulin takes place in endoplasmatic reticulum of beta pancreatic cells. In addition, C-peptide is stored in Golgi secretory granules and is cosecreted in equimolar amounts into the blood stream together with insulin in response to the glucose stimulation [1, 2]. Amino acid sequences of C-peptide are in different species relatively variable. Nevertheless, C-peptide has several conserved sequences, for example, N terminal acidic region, glycine-rich central segment, and C-terminal pentapeptide [3]. Despite the first reports describing C-peptide as a peptide with little or no biological activity, recent data reports binding of radioactive labelled C-peptide on the cell membranes [4]. Other studies show binding effects stimulating Na-K-ATPase. C-terminal pentapeptide gives full replacement of the entire molecule, which is similar to other peptides with hormone function like gastrin and cholecystokinin [5, 6]. The receptor stays unknown but there is a lot of data demonstrating C-peptide biological effects by activating different signalling pathways, for example binding to pertussis-toxin-sensitive G_i-coupled receptor on Swiss 3T3 fibroblasts [7, 8] or activating p38 protein kinase pathway in mouse lung capillary endothelial cells [9]. The approach of Luppi et al. detected C-peptide in early endosomes which can be signalling station in the cell, though C-peptide might achieve its cellular effects [10].

There is a certain controversy regarding reported effects of the C-peptide. Its beneficial effects have been demonstrated in long-term complication in type 1 diabetes. Substitution of C-peptide in type 1 diabetes improves glomerular hyperfiltration, hypertrophy, and proteinuria [11–14]. In contrast to this, C-peptide in type 2 diabetes shows proinflammatory and proatherogenic effects [15, 16]. The aim of this paper focuses on the proinflammatory effects of C-peptide and its potential importance in atherosclerosis in diabetic subjects.

2. Atherosclerosis Is an Inflammatory Disease

Atherosclerotic lesions are molecular and cellular responses in the vessel wall that have been described as inflammatory disease [17]. Endothelial dysfunction is an early event in atherosclerosis and an important feature of glucose intolerance, diabetes, obesity, and dyslipidemia, as well as a major component of cardiovascular disorders, including hypertension and atherosclerosic diseases [18]. The atherosclerotic plaque consists of necrotic core, calcified regions, foam cells with accumulated lipids, inflamed smooth muscle cells, endothelial cells, lymphocytes, and leukocytes [17]. Minimally oxidised LDL in blood can release bioactive phospholipids that can activate vascular endothelial cells to express leukocyte adhesion molecules, such as vascular cell adhesion

molecule-1 (VCAM-1) or intercellular adhesion molecule-1 (ICAM-1) [19]. Highly oxidised LDL can be recognised by monocytes scavenger receptor to be transformed into foam cells (Figure 1). Minimally oxidised LDL-induced expression of adhesion molecules induces initial step in atherosclerosis, leukocyte recruitment, and rolling on the endothelium. Moreover, activated endothelium expresses selectins, monocyte chemoattractant protein-1 (MCP-1), RANTES, and fractalkine, which allow leukocyte adherence to the endothelium [20]. Chemokines are small proteins, and their primary function is activation of specific pertusis-toxin sensitive G-protein-coupled receptors, which results in migration of inflammatory cells [21]. Monocytes and T lymphocytes are migrating into the intima of the vessel wall. Monocytes are expressing scavenger receptor and toll-like receptor, which mediate differentiation into foam cells. These cells in addition play central role in atherosclerotic plaque formation [22, 23]. In atheroma activated macrophages release IL-6, TNFα, MIF, and other proinflammatory cytokines and chemokines as well as nitric oxide. This proinflammatory response promotes replication of smooth muscle cells from the media and formation of extracellular matrix [17].

T lymphocytes are entering the subendothelial space as naïve T0 cells. Family of T cell chemoattractants such as IP-10 can in the same way regulate lymphocyte recruitment into the atheroma [24]. Smooth muscle cells are producing extracellular matrix within the vessel wall and in response to atherogenic stimuli they can modify the type of matrix produced. Further, the type of matrix affects the lipid content of the plaque and the proliferative index of the cells attached to the plaque [25]. T lymphocytes release interferon-γ (IFN-γ) into the plaque, which might block the collagen synthesis in SMC and decrease their ability to renew the collagen. Degradation of extracellular matrix allows penetration of SMCs through elastic laminae and enables plaque to grow [26]. Activated macrophages secrete proteolytic enzymes and matrix metalloproteinases, and that can lead to degradation of the matrix complex of the plaque and destabilisation of the atheroma with increased risk for plaque rupture and can lead to acute clinical events such as myocardial infarction and stroke [27].

2.1. Proinflammatory In Vitro Effects of C-Peptide. Individuals with diabetes have increased risk of coronary heart disease compared with nondiabetic individuals, and the risk of cardiovascular deaths is as high as in nondiabetic individuals with previous myocardial infarction [28].

Marx et al. reported deposition of C-peptide in the subendothelial space in carotid artery in diabetic subjects [15]. In some of the subjects, deposition of C-peptide was found in the media of the artery. In contrast to this, in nondiabetic patients deposition of C-peptide has not been found. All the 21 subjects involved in the study had deposition of C-peptide, with 77% of them also having infiltration of monocytes and just 57% infiltration of T lymphocytes [15]. Marx and colleagues used these results to propose the hypothesis that C-peptide may have chemotactic effects on the inflammatory cells and might have a role in atherosclerosis (Figure 1). *In vitro* migration assays performed in modified Boyden chambers reported that C-peptide induces migration of T lymphocytes and monocytes/macrophages in a concentration-dependent manner. These effects were similar to those induced with monocyte chemokine MCP-1 or T-lymphocyte chemokine RANTES [15, 29]. In addition, checkerboard analysis in the same study showed that C-peptide induces chemotaxis rather than chemokinesis [29]. Also there are no migratory effects of C-peptide on B cells or neutrophils [30]. C-peptide stimulates specific intracellular signalling pathways in different cell types [8, 9, 31], for example, Na+/K+ATP-ase [5, 32] ERK kinase, PI-3 Kinase [8, 29, 32, 33], and AKT [8, 32, 33]. In T lymphocyte or in monocytes, C-peptide mediates its chemotactic activity through an as-of-yet- unidentified pertussis toxin sensitive G-protein-coupled receptor with subsequent downstream activation of PI3-kinase γ. Our experiments demonstrated that a specific inhibitor of Src-kinase, PP2, in addition to transfection of Src siRNA, abolished C-peptide-induced T lymphocyte migration, suggesting that C-peptide also signals through this pathway. Besides, experiments showed that PI-3 kinase activation leads to the involvement of small Rho-GTPases, like RhoA, Rac-1, and Cdc42 in these cells. Furthermore in CD4-positive lymphocytes, C-peptide stimulates phosphorylation of PAK (p21-activated kinase), LIMK (LIM domain-containing protein kinase), and cofilin downstream of Rac-1 and Cdc42, leading to cofilin inactivation and actin filament stabilization. Alternatively, C-peptide activates ROCK (Rho kinase) and MLC (myosin light chain) phosphorylation downstream of RhoA, thereby stimulating myosin-mediated cell contraction [30]. These data supported an active role of C-peptide in chemotaxis of inflammatory cells.

C-peptide has an effect on increased microvascular blood flow in patients with type 1 diabetes [34]. Some studies suggest direct role of endogenous insulin and C-peptide in amelioration of endothelial dysfunction [35]. Additionally, C-peptide increases nitric oxide (NO) production through ERK-dependent upregulation of endothelial nitric oxide synthase (eNOS) gene transcription [36].

In addition, C-peptide positively influences the expression of CD34 scavenger receptor in human THP-1 monocytes. These data suggest that C-peptide may also promote the differentiation of monocyte/macrophages towards foam cells, thus representing another potential proatherogenic effect of C-peptide [37].

Further effects of C-peptide have been investigated on the smooth muscle cells, which are important for the development of atherosclerosis. Stimulation with C-peptide induced proliferation of smooth muscle cells in concentration-dependent manner. Walcher and colleagues showed significantly higher production of KI-67 nuclear protein and 3[H]-thymidine incorporation in vascular cells stimulated with C-peptide. This proliferation was similar to those induced by platelet-derived growth factor (PDGF) [33]. Additionally, C-peptide stimulation induces phosphorylation of protein tyrosine kinase Src and PI-3 kinase, which leads to downstream stimulation of MAP ERK1/2 [33]. It is already demonstrated that activation of ERK1/2 is a crucial step in cell proliferation and differentiation [38]. The downstream

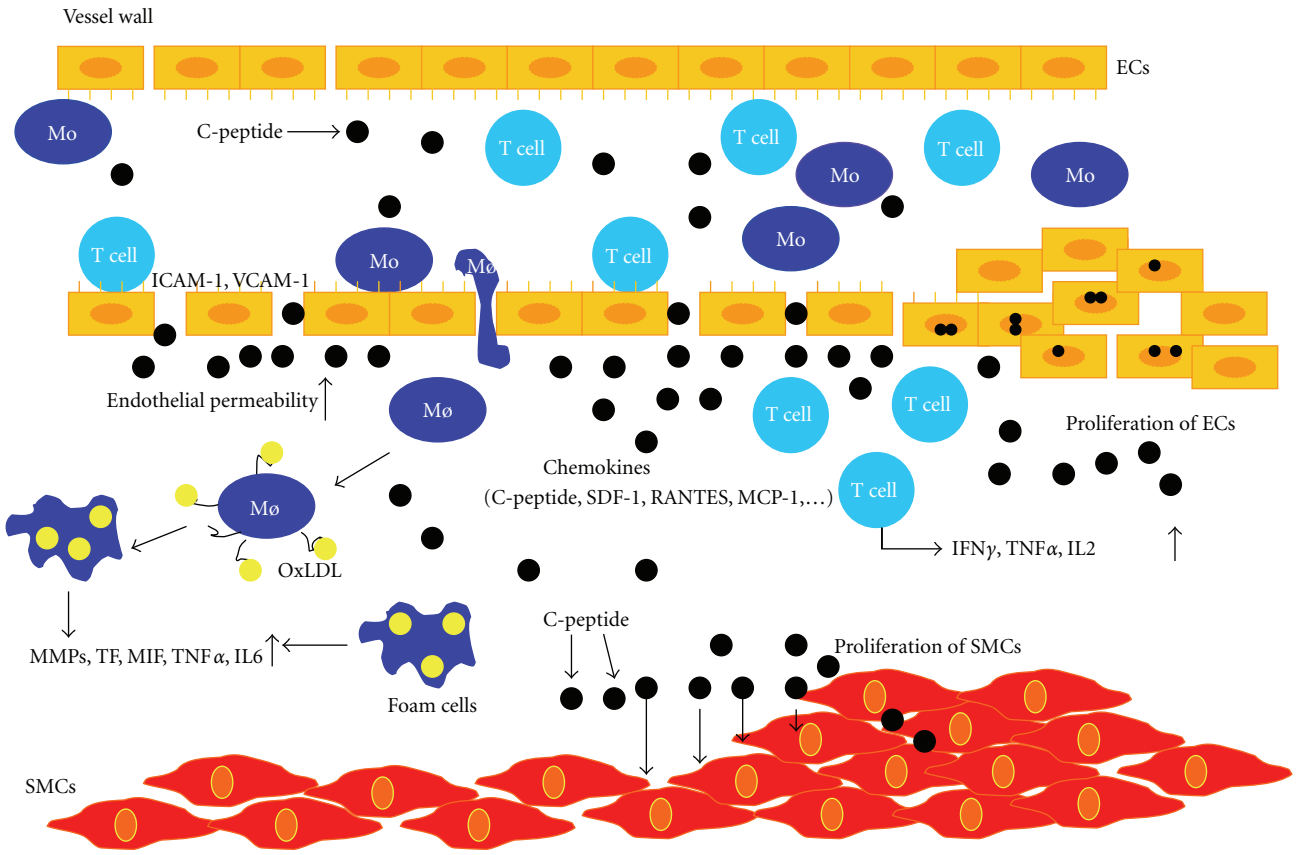

Figure 1: In insulin resistance and early type 2 diabetes insulin levels are increased in circulation. C-peptide levels in blood are increased in equimolar concentration with insulin. C-peptide deposits in subendothelial place in the vessel wall. Deposition is followed by chemotactic effect of C-peptide on the inflammatory cells. It induces migration of monocyte/macrophages and T lymphocytes into the vessel wall. C-peptide has also an effect on the proliferation of smooth muscle cells from the media.

control of VSMC proliferation by extracellular stimuli takes place in mid- to late G_1 phase of the cell cycle, where D-type cyclins promote G_1- to S-phase transition by leading to Rb phosphorylation [39, 40]. Our data showed an increase in cyclin D1 expression, whereas Rb phosphorylation suggested that C-peptide acts via similar signalling pathways [33].

C-peptide mitogenic effects have been detected in other cell types, like endothelial cells, HEK293 cells, and chondrocytes. When endothelial cells were exposed to C-peptide, a significant increase in cell number of 40% was observed [41] (Figure 1). Another group has found that C-peptide stimulates rRNA synthesis, suggesting that the peptide can have proliferative effects and induces expression of 47S in HCS-2/8 chondrocytes derived from a human chondrosarcoma. After 72 hours of exposure to C-peptide, cell counting under a phase contrast microscope and measurement with a cell proliferation kit and BrdUrd staining established that C-peptide exerts proliferative effects on chondrocytes [42].

3. Proinflammatory *In Vivo* Effects of C-Peptide

Observational data from previous studies showed deposition of C-peptide in intima of the carotid artery in diabetic individuals [15]. Further, these data showed that C-peptide induces chemotaxis of inflammatory cells *in vitro* and activation of intracellular signalling pathways [29, 30]. These observations needed *in vivo* experimental model to explore effects of C-peptide in onset of atherosclerosis. To test this hypothesis we applied ApoE-deficient mouse model. The animals were divided into two groups. C-peptide group numbered 18, and placebo 17 mice per group [16]. After subcutaneous injections (200 nmol/injection) of dissolved peptide we identify that C-peptide levels in blood increased 4- to 5-fold compared to basal levels (12.9 ± 1.8 nmol/L compared with 2.7 ± 0.8 nmol/L; C-peptide versus placebo; $P < 0.05$). Simultaneously mice were put on the western type diet to trigger atherosclerosis. Immunohistochemical analysis of the aortic arch showed deposition of C-peptide in the early atherosclerotic plaques. Computer-assisted image quantification revealed significantly higher deposition of C-peptide in treated mice, compared to placebo one (2.1 ± 0.4 versus 0.8 ± 0.1% positive area; $P < 0.01$) treated with water (Figure 2). Similar results were obtained in the aortic root (data not shown). After 12 weeks of C-peptide or water sc injections body weight and lipids (total cholesterol, triglyceride, high density lipoprotein, and low density lipoprotein) did not differ between the two treated

groups. In addition, glucose and insulin levels showed no differences between groups.

Deposition of C-peptide was followed with increased infiltration of inflammatory cells such as monocytes/macrophages in the aortic arch. Moreover, higher deposition of inflammatory cells has been detected in the aortic root (data not shown). Colocalization of C-peptide with inflammatory cells was already demonstrated in early atherosclerotic plaques of diabetic patients [15]. In contrast to this it has been revealed that C-peptide demonstrates antithrombotic effects *in vivo*. Administration of C-peptide in high doses caused delay in arteriolar and venular thrombus growth in normal and diabetic mice [43].

We already know that diabetes accelerates smooth muscle cell proliferation in atherosclerotic lesions and that it correlates with insulin levels [44]. In a study by Walcher et al., authors revealed that the C-peptide acts as a mitogen on the human and rat arterial vascular smooth muscle cells *in-vitro* [33]. Staining for α-actin (Figure 2) in animal model has shown significantly higher content of smooth muscle cells in C-peptide-treated group (C-peptide versus placebo: 4.8 ± 0.6 versus $2.4 \pm 0.7\%$ positive area; $P < 0.01$) as well as a trend towards more KI-67 proliferated cells in C-peptide-treated group.

C-peptide had significantly higher deposition of lipids in aortic arch compared with placebo. Lipid deposition in *en face* preparations of the abdominal and thoracic aorta in C-peptide-treated mice did not reach statistical significance compared to placebo mice (C-peptide versus placebo: $5.64 \pm 0.69\%$ versus $3.98 \pm 0.5\%$; $P = 0.07$) [16]. A possible explanation could be that C-peptide proinflammatory effects obtained in the ApoE-deficient animals were on top of a high-cholesterol diet. Initial aim of this study was to detect deposition of C-peptide in the vessel wall in an animal model without distinguishing metabolic effects. In the future it would be interesting to use a model of diabetes and atherosclerosisprone mice fed with high-cholesterol diet such as ob/ob or LDL−/− mice. Furthermore, nothing is known about C-peptide effects on plaque vulnerability or production of metalloproteinases. Future studies should answer these questions.

4. Discussion

C-peptide is by now identified as a biologically active substance. Many studies initiate C-peptide as an active peptide hormone with important physiological effects, which affects renal, neuronal, and microvascular functions in patients with diabetes [45–48]. C-peptide increases capillary blood flow in type 1 diabetic patients [49], through increased influx of Ca^{2+} into endothelial cells, which facilitate release of NO from the endothelium. Many studies have demonstrated beneficial effects of C-peptide on the long-term complications in type 1 diabetic patients. This could have an important therapeutic implication [11, 12]. For example, decreased blood flow in the extremities might be prevented by C-peptide [50]. Moreover, improvements of endoneurial blood flow and axonal swelling have been also demonstrated by introduction of C-peptide [51]. In numerous studies of type 1 diabetes glomerular hyperfiltration, hypertrophy, and proteinuria have been reduced by C-peptide [13, 14, 52]. C-peptide treatment improves sensory nerve function in early stage of type 1 diabetic neuropathy [47]. The effects of C-peptide on type 2 diabetes as well as on the cell proliferation and apoptosis are very controversial at present. Levels of inflammation in type 1 and type 2 diabetes are still unknown, but it has been found that plasma levels of IL-6 correlate with C-peptide levels and insulin sensitivity [53]. The metabolic syndrome, prediabetes, and type 2 diabetes mellitus accelerate vascular disease and increase development of the disease [54]. At the moment the reasons for the increased predisposition and progression of atherosclerosis in patients with diabetes are unknown. *In vivo* model from Vasic et al.[16] showed increased deposition of C-peptide in early atherosclerotic lesions in ApoE-deficient mice. C-peptide deposition was followed by recruitment of inflammatory cells into the vessel wall and increased infiltration of monocytes/macrophages as well as increased proliferation of smooth muscle cells. These results are also in agreement with *in vitro* data of Swiss 3T3 fibroblasts, where C-peptide has been shown to activate PI-3 kinase [8] as well as increased expression of PPAR-γ regulated CD36 scavenger receptor in human THP-1 monocytes by C-peptide. These results recommend that C-peptide in addition to these effects might promote the differentiation of monocyte/macrophages into foam cells [37]. Our study showed no differences in E-selectin and ICAM-1 levels as well as levels of the inflammatory markers such as TNFα and soluble IL-6. An explanation could be that C-peptide was used in this model on top of the hypercholesterinemic diet. But these data are in contrast to several findings in which C-peptide has anti-inflammatory effects and reduced upregulation of cell adhesion molecules under inflammatory conditions [55, 56]. In mice with endotoxic shock, C-peptide treatment improved survival rate and reduced plasma levels of tumour necrosis factor-alpha (TNFα), macrophage inflammatory protein-1 alpha, and monocyte chemoattractant protein-1 [57].

We already know that the smooth muscle cells and their secreted products are the main components of advanced atherosclerotic lesions [58]. C-peptide deposition was also found in the media in diabetic patients. Moreover, C-peptide induced proliferation of smooth muscle cells *in vitro*, therefore potentially promoting both the development of atherosclerotic lesions and neointima formation after coronary intervention [33]. After vascular injury, smooth muscle cells start to proliferate and migrate into the developing neointima. They develop into the major cellular substrate of the restenotic tissue [59]. *In vivo* studies showed that downregulation of ERK1/2 inhibits early smooth muscle cell proliferation and neointimal thickening in response to arterial injury [60]. In smooth muscle cells C-peptide induces ERK1/2 signalling. Data obtained from ApoE-deficient mice demonstrated significantly higher content of smooth muscle cells in mouse aortic arch, which was followed with higher deposition of lipids in early atherosclerotic lesions in mice treated with high concentrations of C-peptide [16]. In

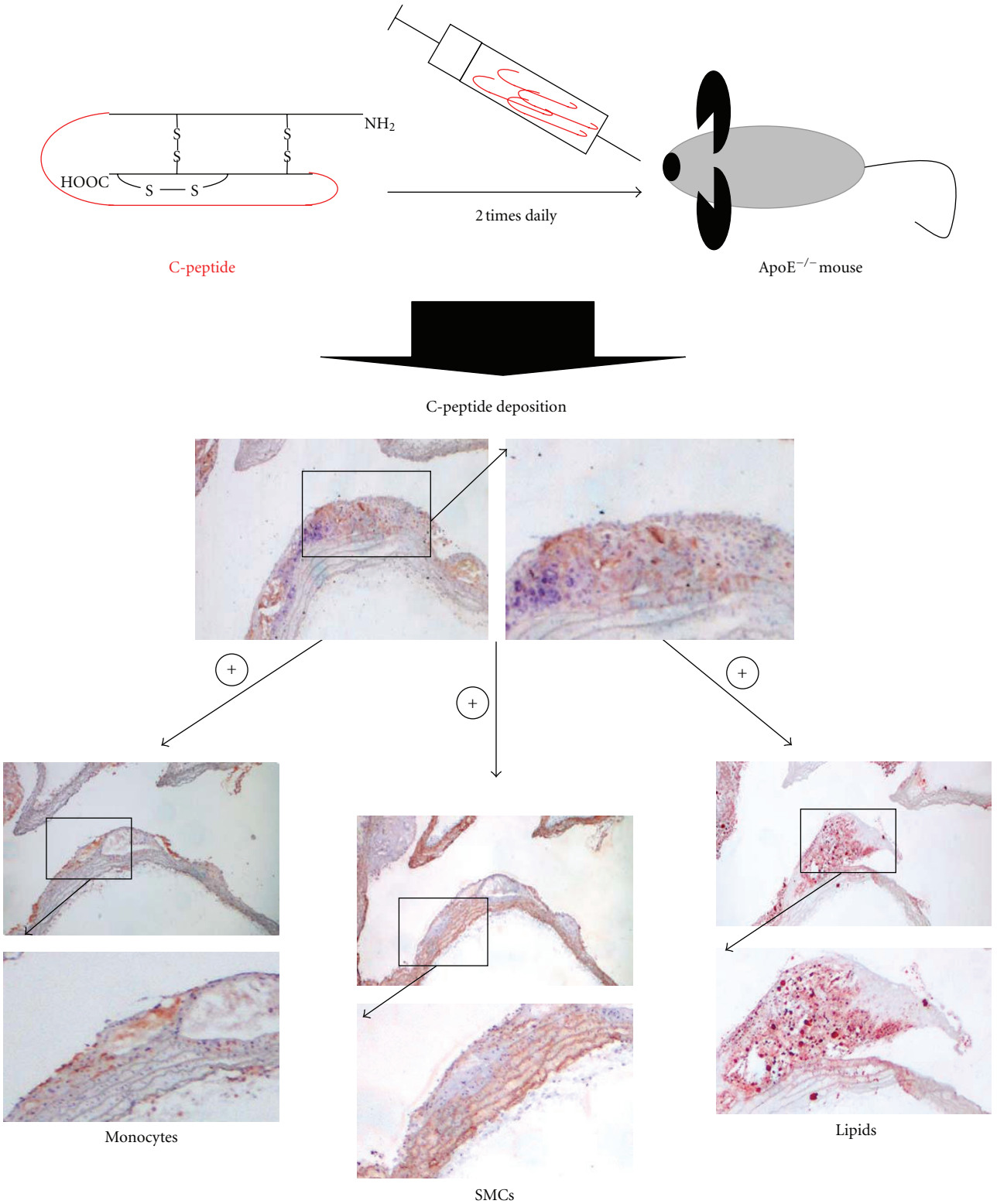

Figure 2: Increased levels of C-peptide in ApoE-deficient mice were established by subcutaneously injections of C-peptide. C-peptide was administrated two times daily by subcutaneous injections for 12 weeks. Deposition of C-peptide in aortic arch has been investigated in mice treated with C-peptide and control mice. Increased deposition of C-peptide in treated mice leads to increased infiltration of inflammatory cells (monocytes/macrophages), increased proliferation of smooth muscle cells from the media, and increased deposition of lipids in aortic arch assessed by immunohistochemistry.

contrast to this, *in vitro* results by Kobayashi revealed that human C-peptide at high concentrations (100 nmol) suppresses the growth of rat SMCs [61]. A recent study by Cifarelli demonstrated that C-peptide significantly decreases caspase-3 activity and upregulated production of the anti-apoptotic factor B-cell CLL/lymphoma 2 (BCL-2) [62]. Anti-inflammatory effects of C-peptide were observed in the study by Chima et al., where C-peptide has been shown to react as inhibitor of lung inflammation following hemorrhagic shock [63].

Conflicting data could be possibly explained with the existence of different circulating insulin and C-peptide levels in diabetes type 2 and diabetes type 1. Most of the studies suggesting anti-inflammatory and antiapoptotic effects of C-peptide performed their experiments on the diverse cell types simulating type 1 diabetes with high glucose levels and low levels of C-peptide where its substitution was beneficial. In regard to this, C-peptide protects endothelial cells from apoptosis and inflammation triggered by high glucose conditions [62]. The situation can be totally different in patients with insulin resistance and type 2 diabetes where high levels of C-peptide could have opposite effects.

A recent study suggests that basal C-peptide levels in type 2 diabetes related to metabolic syndrome correlate to intima-media thickness and C-peptide could be used as surrogate marker of subclinical atherosclerosis [64]. Moreover, Lindahl and colleagues showed that C-peptide stimulates the proliferation of chondrocytes and HEK-293 cells. This regulation of ribosomal RNA means that C-peptide has growth factor activity [42].

In the last few decades C-peptide is presented as an active peptide with diverse effects. Different effects in type 1 and type 2 diabetes seem to be tissue and cell specific. Further work is needed to identify C-peptide receptor and elucidate mechanisms by which C-peptide modulates cell signalling in different cell types.

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Review Article

Pathogenesis of Abdominal Aortic Aneurysms: Role of Nicotine and Nicotinic Acetylcholine Receptors

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Inflammation, proteolysis, smooth muscle cell apoptosis, and angiogenesis have been implicated in the pathogenesis of abdominal aortic aneurysms (AAAs), although the well-defined initiating mechanism is not fully understood. Matrix metalloproteinases (MMPs) such as MMP-2 and -9 and other proteinases degrading elastin and extracellular matrix are the critical pathogenesis of AAAs. Among the risk factors of AAAs, cigarette smoking is an irrefutable one. Cigarette smoke is practically involved in various aspects of the AAA pathogenesis. Nicotine, a major alkaloid in tobacco leaves and a primary component in cigarette smoke, can stimulate the MMPs expression by vascular SMCs, endothelial cells, and inflammatory cells in vascular wall and induce angiogenesis in the aneurysmal tissues. However, for the inflammatory and apoptotic processes in the pathogenesis of AAAs, nicotine seems to be moving in just the opposite direction. Additionally, the effects of nicotine are probably dose dependent or associated with the exposure duration and may be partly exerted by its receptors—nicotinic acetylcholine receptors (nAChRs). In this paper, we will mainly discuss the pathogenesis of AAAs involving inflammation, proteolysis, smooth muscle cell apoptosis and angiogenesis, and the roles of nicotine and nAChRs.

1. Introduction

Abdominal aortic aneurysms (AAAs) usually occur naturally in the infrarenal part in the human abdominal aorta. In men aged 65–80 years, the prevalence of AAAs is between 4% and 8% and approximately six times greater in men than women [1, 2]. An AAA is a permanent localized dilatation of the abdominal aorta (beginning at the level of the diaphragm and extending to its bifurcation into the left and right common iliac arteries in human) that exceeds the normal diameter by 50%, or >3 cm [3].

The primary risk factors of AAAs include family history, smoking, increasing age, male gender, central obesity, and low HDL-cholesterol levels [2, 4]. Hypertension (systolic BP > 160 mmHg, diastolic BP > 95 mmHg) is associated with the AAA risk, but only in women [5]. Diabetes, a well-defined risk factor for atherosclerosis, has been shown to be protective against the AAAs [6–8].

Historically, the AAAs have been considered as a focal manifestation of the advanced atherosclerosis [9]. However, this conventional theory has been challenged by recent evidences: an AAA was a local representation of a systemic disease of the vasculature [10]. There was a lower incidence of AAAs in the individuals suffering from diabetes mellitus that ordinarily considered as the risk equivalent of atherosclerosis [6–8]. The inflammatory cells were recruited into the different sites: the outer media and adventitia of aneurysma, and the intima and subendothelium of atheroma [11–13].

Three key processes contribute to the AAA phenotype: inflammation, proteolysis, and smooth muscle cell (SMC) apoptosis [2]. On the basis of the loss of extracellular matrix especially elastin and accumulation of proteolytic enzymes in the aneurysmal tissues, proteolysis has been regarded as the critical pathogenesis of AAAs [14]. The extracellular matrix degradation by both predominant proteolytic enzymes

MMP-2 and -9, which synthesized and released mainly by the vascular SMCs and infiltrating inflammatory cells such as macrophages, contribute to the anoikis of vascular SMCs. The vascular SMC apoptosis is another critical pathogenesis of AAAs. It has been demonstrated that the decreasing number of the medial vascular SMCs in the vascular wall from the AAAs patients was relevant to apoptosis [15–21]. Degradation of elastin and apoptotic cell death of the medial vascular SMCs destroys the aortic wall integrality, weaken the wall tensile strength, consequently facilitate the development of AAAs. The inflammatory responses in vascular wall play a pivotal role in the MMPs expression and vascular SMC apoptosis. Conversely, the apoptosis and antigen exposure as a result of the extracellular matrix degradation are also likely to contribute to the immune and/or inflammatory responses. Therefore, the initiating factors of AAAs remain mysterious. The mechanism underlying the inflammatory responses in the outer media and adventitia of the vascular wall remains to be well defined. Recent researches have shown that the increased angiogenesis in all layers of the aneurysmal wall is associated with inflammatory responses and related to aneurysmal rupture [22–25].

Cigarette smoking is the irrefutable risk factor of AAAs. It has recently been demonstrated by Stolle et al. [26] that cigarette mainstream smoke enhanced the AAA formation in Ang II-treated apolipoprotein E-deficient mice as a result of the increased proteolytic activity of MMPs. Nicotine, a major alkaloid in tobacco leaves and a primary component in cigarette smoke, plays its pathophysiological roles partly through its receptor—nicotinic acetylcholine receptors (nAChRs). In this paper, we will mainly discuss the pathogenesis of AAAs involving inflammation, proteolysis, vascular SMC apoptosis and angiogenesis, and the roles of nicotine and nAChRs.We made the highlighted change according to the list of references.

2. Nicotine and nAChRs

Nicotine is a principal tobacco alkaloid occurring to the extent of about 1.5% by weight in commercial cigarette tobacco and comprising about 95% of the total alkaloid content. The nicotine in tobacco is largely the levorotary (*S*)-nicotine, only 0.1 to 0.6% of total nicotine content is dextrorotatory (*R*)-nicotine [27].

There are two major types of cholinergic receptors: the muscarinic and the nicotinic. The endogenous ligand, acetylcholine stimulates both receptor types, while the exogenous one, nicotine, preferentially stimulates nAChRs. The nAChRs were firstly identified in excitable cells, but later were identified in many other cell types including vascular and immune/inflammatory cells. There are 17 distinct isoforms (α1–α10, β1–β4, δ, γ, and ε) of the subunits, which form homomeric or heteromeric channels. Among the subtypes, the "muscle-type" nAChRα1, the five polypeptide subunits (α1, β1, δ, and ε in a 2 : 1 : 1 : 1 ratio), and the homomeric "CNS-type", α7-nAChRs, have been identified in a variety of non-neuromuscular cell types such as vascular ECs, vascular SMCs, smooth muscle specific α-actin positive myofibroblasts, T lymphocytes, and macrophages [28–32]. It has been shown that nAChRs, particularly "muscle-type" nAChRs α1 and homomeric CNS-type α7 nAChRs had participated in the pathological processes of atherosclerosis and angiogenesis [33].

3. Nicotine, nAChRs, and Inflammation in AAAs

Inflammation plays a pivotal role in the formation and progression of AAAs and aneurysm rupture [34, 35]. The inflammatory cells including T, B lymphocytes, neutrophils, macrophages, and MCs mostly are recruited into the outer media and adventitia of the aneurysmal wall [13, 22, 24, 36, 37]. Periaortic adipose tissue may also be one of the resident sites of inflammatory cells. Police et al. [38] have demonstrated that the increased number of macrophages in periaortic adipose tissue surrounding the abdominal aortas of Ang II-infused obese mice was associated with the enhanced AAA formation. The inflammatory cells release not only photolytic enzymes to degrade elastin and other matrix proteins, but also inflammatory and chemotactic factors to recruit more inflammatory cells and stimulate the vascular SMC synthetic phenotype by autocrine/paracrine. In previous studies, macrophages have been frequently examined and shown its indispensability in AAAs. T lymphocytes are not indispensable in the AAAs induced by Ang II in apolipoprotein E-deficient male mice, although which play a dominant role in atherosclerosis [39, 40]. Recently, the role of MCs in the AAA development has also been paid more attention by scientists. The specific granule contents from MCs are very important for the inflammatory cell recruitment, pro-MMP and renin-angiotensin system activation, angiogenesis, and vascular SMC apoptosis [36, 41].

It has been shown by few studies that nicotine played a proinflammatory role in vasculature *in vivo* and *in vitro*. Two *in vitro* experiments have demonstrated that nicotine promoted the VCAM-1 and ICAM-1 expression on human coronary artery endothelial cells and human umbilical vein endothelial cells [42, 43]. In another study, chronic (during 90 days) nicotine exposure enhanced the production of proinflammatory cytokines such as TNFα, Interleukin 1β (IL-1β) by macrophages and upregulates the mRNA expression level of VCAM-1, cyclooxygenase-2 (COX-2), and platelet-derived growth factor β (PDGF-β) in the aortas from low-density lipoprotein receptor-deficient mice [44]. It has been well known that VCAM-1 and ICAM-1 were the key mediators of the inflammatory cell migration and infiltration into vascular wall [45]. Nevertheless, more evidences have demonstrated the anti-inflammatory role of nicotine via nAChRs, that is, the so-called cholinergic anti-inflammatory pathway [46–48]. If a hypothesis that "nicotine can stimulate formation and progression of AAAs through inflammation" is true, is it the best explanation that the prolonged exposure to nicotine may induce desensitization and changes in the expression of nAChRs and thus the beneficial effects of nicotine through its receptors may be halted? [49, 50]. It must be conceded that the AAAs were usually detected in the older people with a longer smoking history [49].

Moreover, it has been indicated by some investigations that the inflammatory mediators including COX-2 and 5-lipoxygenase (5-LO) were also associated with the development of AAAs.

COX-2, a limiting enzyme converting arachidonic acid-into prostaglandin, plays an important role in the inflammatory diseases. In human AAAs, the increased expression of COX-2 is associated with the augmented angiogenesis [51]. King et al. demonstrated the increased expression of COX-2 and the upregulated synthesis of PGE_2 selectively in the aortic aneurismal tissues by exposure to Ang II. The selective COX-2 inhibitor, celecoxib, decreased the incidence and severity of Ang II-induced AAAs in apolipoprotein E-deficient mice and C57BL/6J mice [52]. The above studies indicate that the increased COX-2 expression is one of the pathogenesis of AAA formation. It has been implied by limited studies that nicotine could stimulate the COX-2 expression likely through nAChRs. In human umbilical vein endothelial cells, nicotine increases the COX-2, ICAM-1, and PGE_2 expression through NF-*kappa*B activation which mediated by nAChRs [53]. In gastric cancer, nicotine stimulates the COX-2 expression to trigger tumor cell invasion and angiogenesis through the VEGF activation, which subsequently modulates the MMP activity and plasminogen activators expression [54].

Activation of the 5-LO pathway contributes to the biosynthesis of proinflammatory leukotriene mediators in macrophages, MCs, and other inflammatory cells [55]. 5-LO plays a role in promoting the AAA formation induced by an atherosclerotic diet in apolipoprotein E-deficient mice. 5-LO-positive macrophages localize in the adventitia of the diseased mouse and human arteries in the areas of neovascularization and constitute a major component of the aortic aneurysms. 5-LO deficiency attenuates the aortic aneurysms and reduces the aortic MMP-2 activity and diminished plasma macrophage inflammatory protein-1α (MIP-1α) [56]. It has been recently shown that the mRNA levels for the three key enzymes/proteins in the biosynthesis of cysteinyl-leukotrienes, 5-LO, 5-LO-activating protein (FLAP), and LTC_4 synthase (LTC_4S), were significantly increased in the aneurysmal wall from the human abdominal aortas. 5-LO, FLAP, and LTC_4S proteins express in the media and adventitia and localize in the areas rich in inflammatory cells including macrophages, neutrophils, and MCs. Exogenous LTD_4 increased the MMP-2 and -9 release [57]. Houard et al. have similarly demonstrated that, in the aneurysmal wall of the human abdominal aortas, the leukotriene pathway mainly localized in the macrophage-rich adventitial areas [58]. It has been recently indicated that nicotine could induce the 5-LO expression in colon neoplasm [59]. A hypothesis: "smoking promotes pathogenesis of aortic aneurysm through the 5-lipoxygenase pathway." Had been proposed by Takagi and Umemoto [60] in 2005, but to date, it remains to be demonstrated.

Taken together, it has been demonstrated by compelling evidences that the inflammation in vascular wall is one of the pathogenesis of AAAs. Mediated by the inflammatory cells such as macrophages and MCs and inflammatory mediators including VCAM-1, ICAM-1, COX-2, and 5-LO, the inflammatory responses have a preference for the outer media and adventitia of the aneurysmal wall. Currently, the notion that nicotine promotes the AAA formation by its receptor nAChRs is still not supported by robust evidences. Fortunately, in our recent animal experiment, the AAAs have been successfully induced by both nicotine and Ang II in the older C57BL/6J mice, accompanied with the MC degranulation in the adventitia of the abdominal aortas. Maybe, it will point out a direction for further research.

4. Nicotine, nAChRs, and Proteolysis Induced by MMPs in AAAs

Although the abundant connective tissue proteinases including MMPs (MMP-1, -2, -3, -9, -12, and -13), serine proteases (tissue-type plasminogen activator (t-PA); urokinase-type plasminogen activator (u-PA); plasmin; and neutrophil elastase), as well as cysteine proteases (cathepsin D, K, L, and S) [61] have been described in the human AAA tissues, the most attentions have been kept on the members of the matrix metalloproteinase family [24, 62–65]. Previous studies have focused on the 92-*kD* (MMP-9; gelatinase B) and 72-*kD* (MMP-2; gelatinase A) gelatinase/type IV collagenase, both most prominent elastolytic enzymes secreted by the AAA tissues in organ culture and *in vivo,* which are expressed by macrophages, vascular SMCs, fibroblasts, or ECs, most often in the areas adjacent to the infiltrated inflammatory cells [62, 66–70]. Therefore, it has been shown a close relationship between MMPs and inflammatory responses in the aneurysmal tissues.

The MMPs are a group of zinc-mediated enzymes present in the extracellular matrix. It is a fundamental pathogenesis of AAAs that the increased MMPs in vascular wall degrade all kinds of extracellular matrix proteins, particularly elastin [14, 71]. The MMPs are inhibited by the specific endogenous TIMPs, which comprise a family of four protease inhibitors: TIMP-1, -2, -3 and -4. An imbalance in the proteolytic equilibrium between MMPs and TIMPs is a significant factor of the AAA formation [72]. Elastin and collagens type I/III keep the integrality and elasticity of vascular wall, and resist stretch. Under normal conditions, the content of the proteins keeps balance between degradation and synthesis. But in fact, the balance is principally maintained by the collagen metabolism, because elastin is synthesized and deposited in the early childhood and no further significant synthesis occurs in adult life [73]. The content of collagens type I/III increases *compensatively in* the early stage of the disease, while decreases dramatically in the advanced stage. Degradation of elastin and loss of collagens during the advanced stage destroy the wall integrality and weaken the wall tensile strength, which promotes the development and rupture of AAAs. It is supposed that the degradation of elastin is likely to exert a more significant role in the initiating process of AAAs.

It has been recently demonstrated that cigarette mainstream smoke could enhance the proteolytic activity of MMPs including MMP-2 and -9 induced by Ang II and accelerate both formation and severity of AAAs in the

hypertensive apolipoprotein E-deficient mice, [26] while cigarette smoke extract significantly downregulated TIMP-3 in aortic endothelial cells [74]. Similarly, nicotine increases the MMPs (especially MMP-2 and -9) expression and activities in the vascular wall components including ECs, vascular SMCs and infiltrating inflammatory cells such as neutrophils and macrophages, [54, 75–80] and decreases the expression of TIMP-1, -3, and -4 in osteoblasts [81]. Moreover, the endogenic ligand of nicotine, *α7*-nAChRs, is also involved in the MMP-2 and -9 upregulation in human retinal microvascular endothelial cells [80].

Taken together, nicotine and/or its ligantd *α7*-nAChRs have been involved in the synthesis and release of proteolytic ingredients MMP-2 and -9 and decreased the TIMPs expression *in vivo* and *in vitro*, thus very likely to be involved in the pathogenesis of AAAs.

5. Nicotine, nAChRs, and Medial Vascular SMC Apoptosis in AAAs

Histological examinations of both animal and human experimental AAAs have revealed a paucity of medial vascular SMCs in these specimens which are associated with the SMC apoptosis [15–21]. Vascular SMCs synthesize and release the extracellular matrix proteins including collagens, elastin, glycoproteins, and proteoglycans to provide the mechanical integrality to the vascular wall. In the aortic media, collagens are primarily synthesized by vascular SMCs. Theoretically, a paucity of medial vascular SMCs caused by apoptotic cell death, can reduce the synthesis of extracellular matrix proteins and collagens turnover, eventually attenuate the mechanical properties of the aortic wall and result in the formation and complications of AAAs. Henderson et al. have demonstrated that vascular SMC apoptosis, macrophages, and T lymphocytes coexisted in the aortic mediaand companied by the upregulation of proapoptotic initiators, such as Fas/FasL in the human AAA segments obtained from the patients undergoing open repair [15]. Recently, Yamanouchi et al. [82] firstly reported the direct links between medial vascular SMC apoptosis and pathogenesis of AAAs. In an Ang II-induced aneurysm model in apolipoprotein E-deficient mice, a novel caspase inhibitor Q-VD-OPh, inhibited apoptosis by blocking activation of caspases, drastically reduced the number of infiltrating macrophages and $CD3^+$ T cells and remarkably decreased the interleukin-6 level as well as the elastase activity. These findings suggested that inhibition of apoptosis may attenuate the aneurysm formation not only by preventing the vascular SMC depletion but also by affecting the vascular inflammation and matrix degradation.

In *in vitro* studies, cigarette smoke extract causes apoptosis of aortic SMCs, human umbilical vein endothelial cells, pulmonary artery endothelial cells, and aortic endothelial cells from human and rodent animals [83–86]. Beyond our expectations, as a major ingredient of cigarette smoke, nicotine inhibits apoptosis of aortic SMCs and ECs through nAChRs in several investigations [87, 88]. Anyway, the viewpoint that nicotine is involved in the formation and progression of AAAs through apoptotic mechanism seems to be not supported by existing evidences.

6. Nicotine Stimulates Angiogenesis through *α7*-nAChRs in AAAs

Angiogenesis is the new blood vessel formation from preexisting blood vessels and is a prominent feature in both atherosclerosis and AAAs [23, 25, 89]. In 1996, Thompson et al. [23] had demonstrated that the density of the newly formed vessels was increased in all layers of the aneurysmal wall compared with the control samples. The degree of neovascularization is correlated with the extent of the inflammatory infiltration. Angiogenesis is associated with the inflammatory responses in the aneurysmal wall and accelerate the aneurysm rupture [22–25].

Usually, angiogenic stimuli (e.g., hypoxia or inflammatory cytokines) may induce the expression and release of angiogenic growth factors such as vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF). These growth factors stimulate the proliferation of ECs in the existing vasculature and migration through the tissue to form new endothelialized channels [90, 91]. Almost all subunits of nAChRs are expressed on ECs, whereas the most abundant receptor subunit is *α7*-nAChRs [92]. Accumulated evidences have shown that, through excitable *α*7-nAChRs on the plasma membrane of vascular SMCs or ECs, nicotine could stimulate the proliferation and migration of ECs, increase the VEGF and FGF release by vascular SMCs and ECs and promote angiogenesis [29, 54, 79, 93–96]. Moreover, the VEGF receptor and *α*7-nAChRs appear to mediate the distinct but interdependent pathways of angiogenesis [97].

Neovascularization is not the exclusive characteristic of AAAs, which also exists in the atherosclerotic lesion, chronic ischemia, tumor and other illnesses. Therefore, angiogenesis may not be a causative but a "contributing to progression and rupture of aneurysm" factor in the pathological process of AAAs.

It has been recently implied by a chronic nicotine exposure experiment in an hindlimb ischemic mice model that chronic nicotine exposure impaired the angiogenic response to ischemia mediated partly by downregulation of the vascular *α7*-nAChRs, as well as by a reduction in plasma VEGF level [98]. However, because the researches did not assess the functional measures of limb perfusion, the conclusion remains to be further confirmed.

7. Conclusion

It is irrefutable that cigarette smoking is the principal risk factor for AAAs, but as a primary ingredient of cigarette smoke, nicotine, which has role in AAAs is undefined. The increased MMPs expression and degradation of all kinds of extracellular matrix proteins, particularly elastin, in the aneurysmal wall, has been demonstrated by compelling evidences, and nicotine may be involved in the process. The angiogenesis stimulated by nicotine may be the important factor for the progression or rupture of AAAs rather

than the causative factor. Inflammation and apoptosis of vascular SMCs, two important pathogenesis of AAAs, are seemingly irrelevant to nicotine. Whether nicotine is the key component in cigarette smoke promoting the formation and progression of AAAs remains equivocal. In a chronic nicotine exposure experiment during 90 days, the inflammatory responses in the aorta are enhanced [44]. It may be explained as the desensitization and changes in the expression of nAChRs and thus the beneficial effects of nicotine through its receptors may be halted [49, 50]. Moreover, the adverse events of nicotine can be attributed to its dose-dependent effects, with the toxic cardiovascular effects at higher doses [49, 93, 99]. Today, cigarette smoking remains a serious social problem. Nicotine replacement therapy is often used in smoking cessation, although there are little evidences on the safety and efficacy of long-term use. Therefore, it is absolutely necessary to clarify the exact roles of nicotine in AAAs, especially the adverse effects of chronic exposure.

Abbreviations

AAA:	Abdominal aortic aneurysm
MMP:	Matrix metalloproteinase
TIMP:	Tissue inhibitors of metalloproteinase
HDL:	High density lipoprotein
nAChRs:	Nicotinic acetylcholine receptors
SMC:	Smooth muscle cell
EC:	Endothelial cell
MC:	Mast cell
CNS:	Central nervous system
AngII:	Angiotensin II
VCAM-1:	Vascular cellular adhesion molecule-1
ICAM-1:	Intercellular adhesion molecule-1
COX-2:	Cyclooxygenase-2
5-LO:	5-lipoxygenase
FLAP:	5-LO-activating protein
PGE_2:	Prostaglandin E_2
MIP-1α:	Macrophage inflammatory protein-1α
LTC_4S:	Leukotriene C_4 synthase
LTD_4:	Leukotriene D_4
VEGF:	Vascular endothelial growth factor
FGF:	Fibroblast growth factor.

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Research Article

Microalbuminuria and sRAGE in High-Risk Hypertensive Patients Treated with Nifedipine/Telmisartan Combination Treatment: A Substudy of TALENT

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Some antihypertensive drugs have also renoprotective and anti-inflammatory properties that go beyond their effect on blood pressure. It has been suggested that microalbuminuria and glomerular filtration rate (GFR) are associated with circulating levels of the soluble form of the receptor, sRAGE (soluble receptor for advanced glycation ends-products). In the present analysis, we used data from the TALENT study to evaluate soluble receptor for advanced glycation end-products (sRAGE) plasma levels in patients with hypertension and high-cardiovascular risk-treated nifedipine and telmisartan in combination. Treatment with nifedipine-telmisartan significantly decreased mean systolic and diastolic ambulatory blood pressure and resulted in a significant increase in sRAGE plasma concentrations after 24 weeks of therapy. We concluded that in hypertensive patients with early-stage renal disease, sRAGE concentrations are not influenced by either microalbuminuria or GFR. Long-term treatment with a combination of nifedipine-telmisartan may have a beneficial effect increasing sRAGE plasma levels, thus exerting an atheroprotective and anti-inflammatory activity.

1. Introduction

Atherosclerosis has been regarded recently as an inflammatory disease. Most cardiovascular risk factors are associated with enhanced vascular inflammatory state [1]. Among these, hypertension has been suggested to have a proinflammatory action by inducing endothelial expression of cytokines [2]. C-reactive protein (CRP), leukocyte adhesion molecules, chemotactic and pro-inflammatory cytokines and heat shock proteins were found to be increased in patients with essential hypertension [3, 4]. Hypertension and cardiovascular disease (CVD) are intimately connected to chronic kidney disease (CKD) [5]. High blood pressure has also been associated with the development of end-stage renal disease [6]. On the other hand, atherosclerosis, considered one of the traditional risk factors for CVD, is more prevalent in CKD patients, at any stage, than in the general population [7]. Several observational studies have suggested that some antihypertensive drugs such as the inhibitors of the renin-angiotensin system (RAS) or dihydropyridine-based calcium antagonists promote normal endothelial function, thus showing anti-inflammatory activity [8, 9]. Due to this specific anti-inflammatory property, the RAS blockade can exert another effect, independently of its hemodynamic action, namely, the reduction of the development and progression of kidney disease [10]. In the setting of nephroprotection, the

beneficial effect of counteracting the RAS system has been suggested to be due, at least in part, to the modulation of the interaction between the receptor for advanced glycation end products (RAGE) and its ligands [11]. RAGE is a multiligand member of the immunoglobulin superfamily of cell surface molecules, which was first described as a receptor for advanced glycation end products (AGEs) [12, 13]. AGEs have a proinflammatory action, and have been related to endothelial dysfunction, arterial stiffening and hypertension [14]. Furthermore, the interaction between RAGE and its ligands is intimately involved in the pathobiology of a wide range of diseases that share common features, such as enhanced oxidative stress, immune/inflammatory responses, and impaired cell functions [15]. RAGE has a secreted isoform, termed soluble RAGE (sRAGE) that lacks the transmembrane anchoring domain and therefore circulates in plasma. Soluble RAGE has AGE-binding properties and acts as a scavenger receptor, preventing the generation of the signalling cascade usually associated with RAGE, thus acting as an inhibitor of AGE-mediated effects [16]. High plasma levels of sRAGE have been reported to be associated with a lower incidence of coronary artery disease (CAD) [17], and low plasma levels of sRAGE have been reported in patients with carotid and femoral atherosclerosis as well as those with metabolic syndrome [18]. Recently, it has been found that sRAGE correlates with the extent of 24-hour albumin excretion, confirming the involvement of the RAGE-ligand axis in the development of diabetic nephropathy [19]. Nevertheless this correlation has not been confirmed by subsequent studies on patients with normal values of glomerular filtration rate (GFR) and therefore the role of sRAGE in the setting of early-stage renal disease has not been fully elucidated [20]. Moreover, a number of drugs recently proved to be able to modulate sRAGE levels [21].

The aims of the present study were (1) to study the association between sRAGE and microalbuminuria in hypertensive patients at high cardiovascular risk and (2) to evaluate the effect of treatment with telmisartan and nifedipine, which are antihypertensive drugs with specific anti-inflammatory effect, on sRAGE levels according to the presence or absence of microalbuminuria.

2. Methods

2.1. Study Population. Participants were derived from the TALENT study (multicenter study evaluating the Efficacy of nifedipine GITS-telmisartan combination in blood pressure control and beyond). TALENT was a multicenter randomized double-blind prospective parallel group (3-arms) trial. It was approved by Ethics Committees/Institutional Review Boards of the centers involved and was conducted according to Good Clinical Practice and under the guiding principles of the Declaration of Helsinki. All individuals provided their informed consent to participate. Included were men and women aged 18–75 years whose office systolic blood pressure was higher than 135 mmHg and with one or more of the following cardiovascular risk factors: type 2 diabetes mellitus (fasting blood glucose ≥126 mg/dL or history of antidiabetic drug use), microalbuminuria, electrocardiographic or echocardiographic evidence of left ventricular hypertrophy and metabolic syndrome. The protocol details and main study results are published elsewhere [22]. Patients with a history of stroke, a transient ischemic attack or myocardial infarction within the previous 12 months, those on angiotensin receptor antagonist or calcium antagonist and those with renal failure (serum creatinine >2 mg/dL) or macroalbuminuria were excluded from the study. Patients were randomized to one of the following treatment groups in a 2 : 1 : 1 ratio: group A received the combination of nifedipine GITS 20 mg/telmisartan 80 mg per day; group B received monotherapy with nifedipine GITS 20 mg/day; group C received monotherapy with telmisartan 80 mg/day. After 8 weeks of treatment, all the 2 monotherapy groups were switched to the combination (telmisartan 80 mg-nifedipine GITS 20 mg per day). From week 16 to week 24, patients were allowed to continue the combination treatment for an additional 8 weeks with the option to uptitrate nifedipine GITS to 30 mg.

To limit any potentially confounding effects due to outlying values, we chose to exclude from our analysis subjects with any CRP values >10 mg/L. We also excluded those with missing serum biomarkers or microalbuminuria analyses at any of the study visit.

2.2. Methods. Frozen blood sample from baseline, 16 and 24 weeks, were simultaneously analyzed at a core facility. As in previous clinical studies involving total sRAGE (esRAGE plus cRAGE), we determined plasma sRAGE levels using a commercially available ELISA kit (Quantikine; R&D systems) according to the manufacturer's instructions. Briefly, a monoclonal anti-sRAGE antibody was used to bind plasma sRAGE, and bound sRAGE detected with a horseradish-peroxidase-linked polyclonal antibody specific for the extracellular domain of RAGE. After washing, a hydrogen peroxide solution was added to each well, and optical density at 450 nm was determined with a microplate reader (Multiscan Ex, Thermolabsystems). The intraassay and inter-assay coefficients of variation values were 6% and 8%, respectively and the minimum detectable dose of RAGE ranged from 1.23–16.14 pg/mL. The presence/absence of microalbuminuria was detected by local laboratories by the CLINITEK Status Analyzer, Siemens. GFR was estimated using the Modification of Diet in Renal Disease Study equation, equation 7, derived by Levey et al. [23].

All measurements were performed without knowledge of the treatment assignment.

2.3. Statistic. Continuous data are reported as mean ± SD along the paper, even in included tables, if the data were skewed. Categorical data are presented as per group percentages. Differences between subgroups were evaluated using Student's t-test for the normally distributed ones. If data were skewed, the logarithmic transformation was done to make the data valid for Student's t-test. Differences in categorical data were compared using the chi-square test. Simple (univariate) linear regression model was adopted to analyze the association of sRAGE with GFR. A two-tailed P value

less than 0.05 was considered to be statistically significant. The relationship between two variables was tested using the Pearson's correlation coefficient.

3. Results

Among the 405 subjects included in the TALENT study, inflammatory markers and microalbuminuria data were available for 262 patients (173 males and 89 females, mean age 59 ± 9 years). Among those patients, 99 (38%) had a positive result at the microalbuminuria test (30–300 mg/24 h), while the remaining 163 (62%) had a negative result (<30 mg/24 h). The mean GFR value of our cohort at the recruitment was 93 ± 23 mL/min.

No significant differences were found in plasma sRAGE levels concerning sex of the participants (141 pg/mL versus 138 pg/mL, P = ns), according to the diabetic (134 pg/mL versus 152 pg/m) and smoking status (139 pg/mL versus 142 pg/mL, P = ns) or statin use (145 pg/mL versus 140 pg/mL, P = ns). The log-transformed sRAGE levels showed a positive correlation with serum creatinine (r = 0.14, P = 0.0059) and uric acid (r = 0.122, P = 0.016), but were not significantly correlated with systolic or diastolic blood pressure (r = 0.09 and r = −0.05, P = ns), GFR (r = −0.07, P = ns), and hsCRP (r = 0.05, P = ns) in the entire study cohort.

Baseline characteristics of the patients, according to the presence/absence of microalbuminuria are listed in Table 1. As shown in the Table 1, patients with and without microalbuminuria were similar with respect to age, sex, blood pressure, lipid profile, and GFR values. Plasma concentrations of sRAGE were similar between the two groups. Of interest, baseline plasma concentration of sRAGE was not associated with GFR (r = −0.08, P = ns), both in patients with (r = 0.11, P = ns) and without microalbuminuria (r = −0.06, P = ns).

After 16 weeks of telmisartan-nifedipine treatment according to the study protocol, a significant reduction in ambulatory blood pressure was observed: 24-h mean systolic blood pressure decreased from 137 ± 5 mmHg to 126 ± 5 mmHg (P < 0.001), and diastolic blood pressure decreased from 82 ± 5 mmHg to 75 ± 5 mmHg (P < 0.001). At that time point, the percentage of patients with microalbuminuria decreased from 38% to 29% (P < 0.001), and a trend towards an increase in sRAGE plasma concentration was observed (P = ns).

After other 8 weeks of treatment with the combination telmisartan-nifedipine, the percentage of patients with microalbuminuria decreased to 28% (P < 0.01 from baseline). Treatment with telmisartan-nifedipine resulted in a significant increase in sRAGE values as compared with baseline (log-transformed sRAGE 5.16 ± 0.6 versus 5.13 ± 0.6, P < 0.05). A similar increase in sRAGE plasma concentrations was found in patients with and without microalbuminuria (0.03 ± 0.2 versus 0.05 ± 0.2 P = ns).

Furthermore, we studied the relationship between the change in sRAGE values and ambulatory blood pressure from baseline to week 24. We observed that the increase in log transformed sRAGE concentrations was not proportional to the decrease in 24 h mean systolic nor diastolic blood pressure, as shown in Figure 1.

Table 1: Baseline characteristics of patients with and without microalbuminuria. Continuous data are reported as mean ± SD or median (interquartile range), categorical data are presented as per group percentages.

Variable	With microalbuminuria N = 99	Without microalbuminuria N = 163	P value
Age, years	60 ± 10	58 ± 10	ns
Males	62%	68%	ns
Diabetes mellitus	59%	61%	ns
Mean 24-h SBP, mmHg	137 ± 5	136 ± 5	ns
Mean 24-h DBP, mmHG	84 ± 5	85 ± 5	ns
Total cholesterol, mmol/L	5.06 ± 0.96	5.14 ± 1.14	ns
HDL cholesterol, mmol/L	1.24 ± 0.31	1.21 ± 031	ns
LDL cholesterol, mmol/L	3.1 ± 0.85	3.15 ± 1.01	ns
Triglycerides, mmol/L	1.74 ± 1.1	1.65 ± 0.95	ns
Body mass index, Kg/m^2	29 ± 3	29 ± 3	ns
Smokers, %	56%	48%	ns
Creatinine, μmol/L	82.2 ± 19.4	82.2 ± 19.4	ns
GFR mL/s/1.73 m^2	1.55 ± 0.38	1.57 ± 0.45	ns
Log-transformed sRAGE	5.12 ± 0.6	5.14 ± 0.6	ns

SBP: systolic blood pressure; DBP: diastolic blood pressure; GFR: glomerular filtration rate.

4. Discussion

According to the 2010 United States Renal Data System report, CKD patients, with any stage of CKD, have higher rates of hypertension, and the prevalence of hypertension increases with the stage of kidney disease [24]. Cardiovascular disease, as well, is tightly bound to impairment of kidney function, as demonstrated by Anavekar and colleagues in the VALIANT study, being the risk of death from any cardiac event inversely associated with the glomerular filtration rate, making even mild kidney dysfunction a major unconventional risk factor for cardiovascular complications [25]. RAGE signalling has been suggested to play a pivotal role in inducing inflammatory process and endothelial dysfunction, that characterize both diabetic and nondiabetic atherosclerosis and its clinical manifestations [15]. Microalbuminuria, which is an independent risk factor for renal and cardiovascular disease, is a feature of hypertension, metabolic syndrome, and type I diabetes mellitus and is supposed to be associated with higher values of sRAGE [19, 26]. In a subanalysis of the TALENT study, we found that the presence of microalbuminuria does not

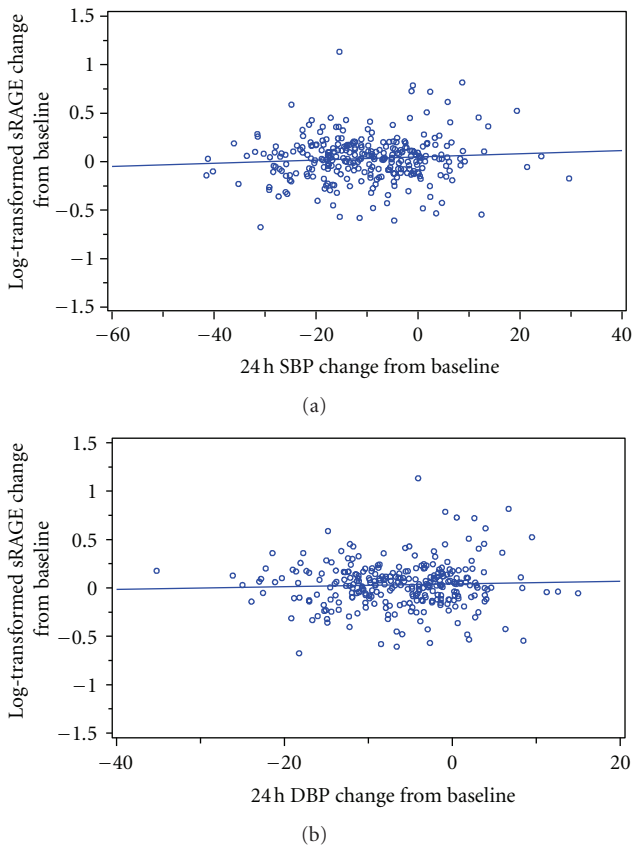

Figure 1: Log-transformed sRAGE by 24 hour systolic (a) and diastolic (b) blood pressure change from baselin at week 24.

affect baseline sRAGE plasma concentrations. Furthermore, antihypertensive treatment with the combination nifedipine-telmisartan increased sRAGE values regardless of the presence of microalbuminuria. Even if the TALENT study was not designed to evaluate effect of the intervention on sRAGE levels, we can hypothesize that this is related to a specific effect of nifedipine-telmisartan on endothelial function.

Angiotensin II activates NAD(P)H oxidase which stimulates the production of reactive oxygen species; this increases the function of the proinflammatory transcription factor nuclear factor-κB which controls the expression of proinflammatory cytokines. Angiotensin II also stimulates growth factors, extracellular matrix protein synthesis, and matrix metalloproteinases, thereby promoting proliferative and fibrotic mechanisms involved in vascular remodelling. Telmisartan has an additional specificity of action that relates to a partial peroxisome proliferator-activated receptor-γ (PPAR-γ) agonism. In vitro studies have recently shown that through this peculiar function of PPAR-γ, telmisartan inhibits superoxide generation and monocyte chemoattractant protein-1 (MCP-1) gene expression in mesangial cells, thus exerting an anti-inflammatory activity against AGE-mediated kidney damage [27].

Long-acting CCBs exert antioxidant effects that protect LDL and membrane lipids from oxidation, this effect being independent of calcium channel modulation [9]. It has been found that nifedipine inhibits the TNF-α-induced generation of reactive oxygen species and the subsequent MCP-1 and VCAM-1 expression in human cultured endothelial cells by suppressing NADPH oxidase activity. Furthermore, nifedipine is known not only to upregulate endothelial nitric oxide synthase expression, but also to increase NO bioactivity via superoxide dismutase induction in endothelial cells [28].

As a consequence, both telmisartan and nifedipine share the potential to exert specific anti-inflammatory actions that go beyond their class effects. Evidences from preclinical and clinical studies suggest that the complementary mechanisms of action of these drugs provide greater efficacy when used in combination. TALENT confirms that the duration of treatment is a key factor: as a matter of fact, the increase in sRAGE was significant only after 24 weeks of treatment whereas blood pressure reduction was already evident after two weeks [22].

Our observation of a significant increase in sRAGE values during nifedipine-telmisartan treatment corroborates the possible role of these drugs in influencing RAGE-ligands signaling as already shown also in other studies [27, 29]. The biological consequence of an increased amount of endogenous sRAGE is an anti-inflammatory and antithrombotic effect. Interactions between RAGE and its ligands result in enhanced transcription and production of various pro-inflammatory mediators via activation of nuclear factor-κB. These RAGE-dependent pro-inflammatory genes include intracellular adhesion molecule-1 (ICAM-1), VCAM-1, E-selectin, TNF-α, interleukin-1, interleukin-6, and cyclo-oxygenase-2. Therefore, ligand-RAGE binding is thought to be involved in the formation, progression, and instability of atherosclerotic plaques with subsequent macrovascular complications. Since sRAGE is the circulating isoform of RAGE which has been identified and theorized to competitively prevent the binding of AGEs to the transmembrane form of RAGE, drugs that increase sRAGE plasma concentrations may have the effect of attenuating atherosclerosis. This hypothesis has been supported by animal models in which administration of sRAGE to mice retarded the progression of atherosclerosis [30].

Microalbuminuria is a marker of microvascular endothelial dysfunction, and reduction of microalbuminuria could reflect a better haemodynamic situation, namely, a decreased shear stress, that ameliorates the endothelial performance and modifies sRAGE levels, thus reducing the cardiovascular risk [31]. In the TALENT study, we found that sRAGE plasma levels were not affected by the presence or absence of microalbuminuria. Our data are consistent with previous observations on diabetic Chinese patients with early stage-renal disease [32]. On the contrary, in patients with advanced degree of kidney dysfunction, documented by a reduced GFR and sRAGE concentrations were found to be positively associated with increased 24-hour albumin excretion [33]. The mean value of GFR in our study population was 93 mL/min, confirming that our patients can be considered at an early stage of renal disease, since patients with elevated creatinine plasma levels and those with macroalbuminuria (spot urine albumin/creatinine ratio >300 mg/g) were excluded from the study. Therefore, the apparent discrepancies between

the studies could be explained by the different severity of renal impairment.

Our study did not find any associations between sRAGE and GFR values. A recent study by Basta and colleagues identified a GFR of approximately 60 mL/min as a threshold below which sRAGE accumulates [34]. Thus, the lack of association between GFR and sRAGE plasma levels in our study could be explained by the GFR higher than 90 mL/min.

The role of calcium antagonists on the kidney with previous formulations of the drugs [35, 36] has been quite controversial; however, more recent controlled trials, including those with nifedipine GITS, demonstrated an effective renal protection as long as blood pressure is also controlled [37]. In addition, a recently published paper by Nakamura and colleagues has demonstrated the beneficial effect of a calcium channel blocker on sRAGE levels in a small cohort of nondiabetic patients with early-stage kidney disease [38]. Our data are in line with the forementioned study and have the advantage of having been achieved on a larger population. The combination of a calcium antagonist and an angiotensin receptor blocker has a strong rationale and should be more effective than the monotherapies as demonstrated in the TALENT study [22].

A limitation of our study is that the assay used to assess sRAGE levels is unable to distinguish among different circulating isoforms of sRAGE. Therefore, since the proportion of different isoforms of sRAGE could differ among individuals, our study cannot clarify the pathophysiology of the regulation of sRAGE production in patients with and without microalbuminuria, which would be the subject of a different study.

In conclusion, our study suggests that in high-risk hypertensive patients plasma sRAGE levels do not correlate with microalbuminuria as long as the GFR is preserved. In this specific population, the increase of sRAGE plasma concentration and the reduction of microalbuminuria may indicate benefits that go beyond blood pressure reduction obtained by a nifedipine-telmisartan combined treatment.

Conflict of Interests

The authors declare that they have no conflict of interests.

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