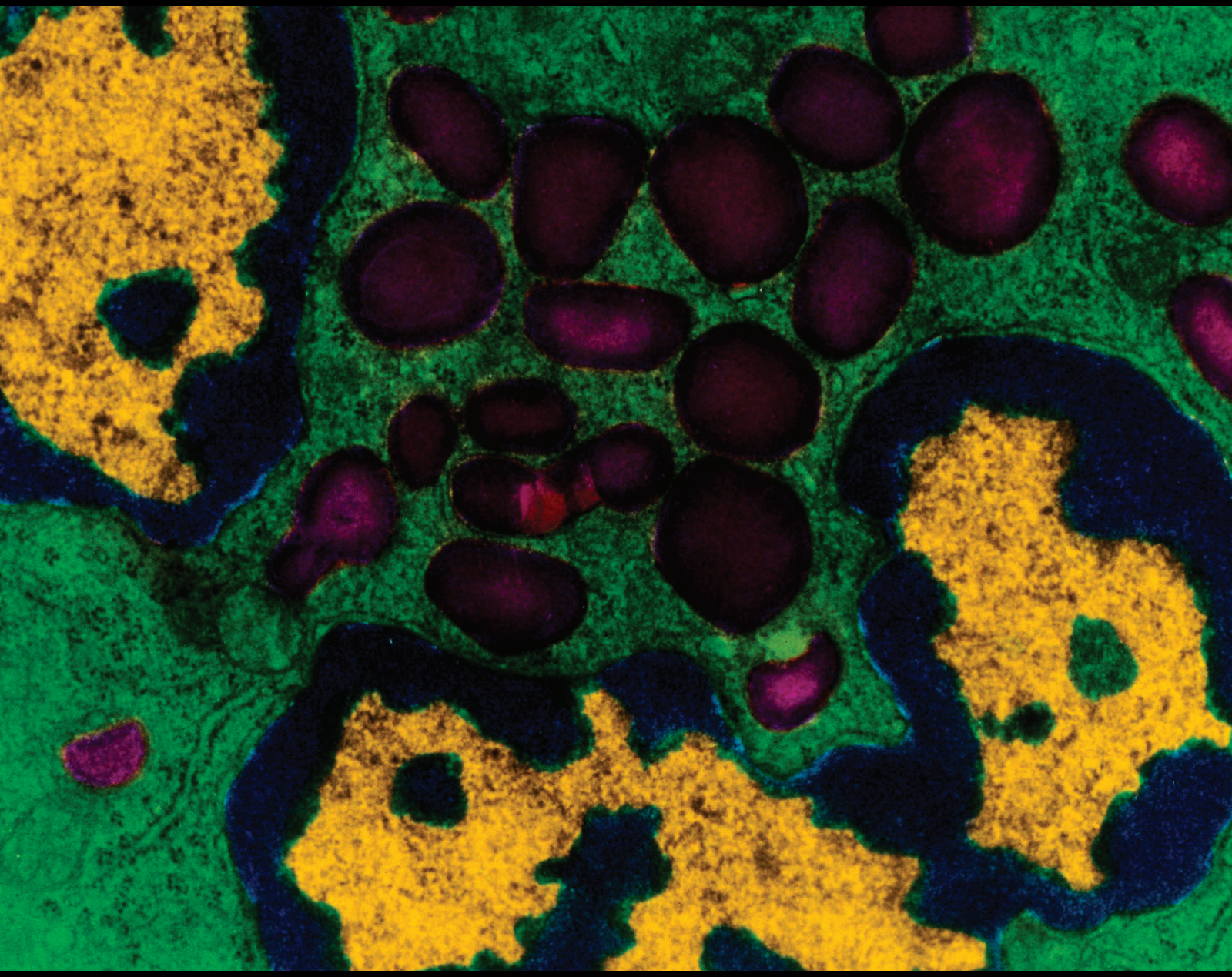
Mediators of Inflammation

Interplay of Inflammation, Immunity, and Organ-Specific Adiposity with Cardiovascular Risk

Guest Editors: Massimiliano M. Corsi Romanelli, Gianluca Iacobellis, and Massimo Locati



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Editorial Board

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Editorial

Interplay of Inflammation, Immunity, and Organ-Specific Adiposity with Cardiovascular Risk

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Adipose tissue is a metabolically active organ with anatomical and functional contiguity to many different organs and also myocardium. Under physiological conditions, adipose tissue displays biochemical properties; under pathological circumstances, adipose tissue can affect the heart and vessels through vasocrine and/or paracrine secretion of proinflammatory molecules, such as cytokines, chemokines, and adipokines [1].

Obesity can be considered a state of chronic, low-grade inflammation [2]; particularly visceral adipose tissue (VAT) seems to be an active compartment in proinflammatory molecule secretion [3].

In this special issue, five papers are devoted to explain the linkage between inflammation and organ specific adiposity correlated with cardiovascular risk.

The paper of D. Lio et al. focused attention on a Toll Like receptor, the TLR-4, and its importance in immunity and cardiovascular risk. Moreover, environmental factors play an important role in the interplay of inflammation, organ adiposity, and cardiovascular risk. In fact, M. Greco et al. propose the Mediterranean diet as a scavenger for it.

E. De Falco et al. proposed that adiponectin plays an important role in anticoagulated patients.

M. Bova et al. describe an association between TGF-β and thoracic aortic aneurysm. Last but not least, a careful analysis of correlation among carotid stenosis and RANKL is presented by S. Lenglet et al.

We hope that readers will find in this special issue not only accurate data and update review on the mechanisms of interplay of immunity, organ adiposity, and cardiovascular risk, but also important questions to be resolved such as innate immunity role [4], prevention, the effect of health care system, and the role of new molecules.

Massimiliano M. Corsi Romanelli
Gianluca Iacobellis
Massimo Locati

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Research Article

Can the TLR-4-Mediated Signaling Pathway Be "A Key Inflammatory Promoter for Sporadic TAA"?

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Thoracic aorta shows with advancing age various changes and a progressive deterioration in structure and function. As a result, vascular remodeling (VR) and medial degeneration (MD) occur as pathological entities responsible principally for the sporadic TAA onset. Little is known about their genetic, molecular, and cellular mechanisms. Recent evidence is proposing the strong role of a chronic immune/inflammatory process in their evocation and progression. Thus, we evaluated the potential role of Toll like receptor- (TLR-) 4-mediated signaling pathway and its polymorphisms in sporadic TAA. Genetic, immunohistochemical, and biochemical analyses were assessed. Interestingly, the rs4986790 TLR4 polymorphism confers a higher susceptibility for sporadic TAA (OR = 14.4, $P = 0.0008$) and it represents, together with rs1799752 ACE, rs3918242 MMP-9, and rs2285053 MMP-2 SNPs, an independent sporadic TAA risk factor. In consistency with these data, a significant association was observed between their combined risk genotype and sporadic TAA. Cases bearing this risk genotype showed higher systemic inflammatory mediator levels, significant inflammatory/immune infiltrate, a typical MD phenotype, lower telomere length, and positive correlations with histopatological abnormalities, hypertension, smoking, and ageing. Thus, TLR4 pathway should seem to have a key role in sporadic TAA. It might represent a potential useful tool for preventing and monitoring sporadic TAA and developing personalized treatments.

1. Introduction

Heart and vascular system, including particularly the large elastic arteries, that is, the aorta, shows with advancing age a multitude of changes at different structural and functional levels [1–5]. As a result, vascular remodeling (VR) and medial degeneration (MD) occur [2, 4]. At the macroscopic level, these pathological entities induce weakening of aorta wall and a progressive stiffness [2, 4]. Endothelial dysfunction, increased oxidative stress, inflammatory reaction, inflammatory cell infiltration in aortic wall, apoptosis of vascular smooth muscle cells (VSMC)s, degeneration of aortic media, and elastin fragmentation and degradation represent their microscopic alterations [2, 4]. In turn, they can degenerate in aortic dilatation and aneurysm and increase the onset risk for complications, that is, aortic dissection and rupture. VR and MD are the typical pathological entities of several aorta diseases, including the inherited syndromic and familial forms of thoracic aortic aneurysm (TAA) and the sporadic forms [6]. Among these, sporadic TAA is becoming a common and serious health risk because of growing enhance of old people in Western populations [7, 8]. Aged population shows an increased incidence for sporadic TAA with advancing of years, as recently reported by epidemiological studies executed in geographic regions with stable populations with little out- or in-migration, such as in Minnesota and Sweden [9, 10]. Another determining factor related to the population ageing is the increased number of hypertensive individuals

[11]. Hypertension is, indeed, a widely prevalent and important risk factor for cardiovascular diseases, including sporadic TAA, as established by recent guidelines [11].

Sporadic TAA is considered a pathology by unclear mechanisms [12]. However, current research's interest is enormously increasing, even if the literature data about its genetic, molecular, and cellular mechanisms are inconsistent. In addition, it is growing the opinion to consider thoracic aortic aneurysms, and particularly the sporadic forms, as immune/inflammatory diseases with a strong genetic component [13]. An active participation of both innate/inflammatory and clonotypic responses has been evidenced. He and colleagues observed an infiltration of inflammatory/immune cells in the media and adventitia from aorta samples of patients with sporadic TAA [14, 15]. Accordingly, we detected a significant high number of CD3+CD4+CD8+CD68+CD20+ cells in tissue aorta samples from patients with Stanford type A aortic dissection (TAAD). A significant role of inflammatory variants in the TAAD risk was also identified in our study [16]. Thus, chronic inflammation might contribute to the pathogenesis of sporadic TAA. This also leads to supposing that sporadic TAA may be the result of a complex combination of factors, including a high genetic propensity, epidemiology factors, age-related vascular alterations, hemodynamic stress, chronic inflammation, and aortic injury. In this complex scenario, the identification of the pathways activated by these chronic stressors might be crucial, in order to translate experimental data in clinical new personalized measures of prevention, diagnosis, treatments, and management.

In consistency with this suggestion, we propose that sporadic TAA might be the consequence of an active stimulation of a particular inflammatory signaling pathway, the Toll-like receptor (TLR)-4-mediated signaling pathway, able to recognize both pathogens and endogenous ligands. An increasing number of studies underlines the weight of TLR4-mediated signaling pathway in several cardiovascular diseases (CVD)s [23, 30–36]. In addition, its strong role in age-related aorta dysfunction, aneurysm's onset, and its complications (dissection or rupture) recently is emerging. A histopathological study demonstrated the TLR4 expression's profile in all cells of arterial wall, and particularly in endothelial cells (EC)s and VSMCs. It also evidenced its functional importance in mediating physiological aorta homeostasis and maintaining of protection against pathogens and damaging cell molecules, as well as in inducting pathological aorta phenotypes [23]. Recent experimental investigations in animal and *ex vivo* models also emphasises its role in the vascular aorta alterations (VR and MD) and their complications, that is, sporadic TAA, by inducing or modulating increased expression and activation of endothelium dysfunction and remodeling aorta pathways (i.e., angiotensin converting enzyme (ACE), endothelial oxide nitric synthase (eNOs), and metalloproteinase (MMP) pathways) [37–42]. In addition, genetic variants of TLR4-mediated signaling pathway have been associated with the susceptibility for several CVDs [30–36]. In particular, polymorphisms of TLR4 gene (NM-138554.1), and particularly the rs4986790 TLR4 polymorphism, have been associated with the risk for several CVDs and other age-related diseases (i.e., Alzheimer disease, prostate cancer, and diabetes), even if contrasting results have been reported [30, 32, 36, 43].

In the light of this current evidence, we sought in the present study to investigate the potential role of TLR4-mediated signaling pathway (promoter) in sporadic TAA. Precisely, we analysed the weight of ten genetic variants related to TLR4-mediated signaling pathway in disease susceptibility, evocation of aorta abnormalities, systemic inflammation (arterial-associated senescence secretor phenotype-AASSP), and vascular biological ageing. Thus, associations between their combined risk genotypes and typical pathological tissue phenotypes, systemic inflammatory mediator levels, inflammatory/immune infiltrate, apoptosis of VSMC cells, tissue MMP-9 amounts, and attrition of telomeres were also evaluated.

2. Materials and Methods

2.1. Patient Population. Our study included 161 individuals (127 men (78%) and 34 (22%) women; mean age: 63 ± 10.7) from Western Sicily enrolled precisely from January 2004 to July 2008 at time of their admission to Cardiac Surgery Unit of Palermo University Hospital. They were affected by sporadic TAA and diagnosed through imaging technologies (i.e., ECHO, CT and MRI) and with localization essentially in ascending aorta (precisely in aortic sinus and tubular portion and sometimes only in tubular portion) and in aortic bulb or both (see Table 1). For the patient's selection, histopathological analyses and exclusion criteria for syndromic and familial forms (e.g., Marfan and Ehler's Danlos syndromes) and autoimmune connective tissue disorders were utilised. According to 2010 guidelines, elective or acute surgical treatment (using wheat operation, Bentall-De Bono and Tirone David surgical techniques, whenever possible) and consequent resection were performed after evaluation of aortic transverse diameter sizes [44, 45]. Their evaluations are reported in detail in the paragraph "Evaluation of aortic transverse diameter sizes" in Supplementary Material available online at http://dx.doi.org/10.1155/2014/349476. The mean of these values was reported in Table 1.

Medical histories pertinent to aortic disease were obtained from patient's medical records. Thus, demographic and clinical features, comorbidity conditions, and pharmacological treatments were collected (see Table 1).

2.2. Control Population. The control group consisted of 128 subjects (61 (47%) men and 67 (53%) women; mean age: 61.08 ± 5.83 years), belonging to the same ethnic group of patients, in order to include in the study a very homogenous population. Ethnicity was confirmed, since parents and grandparents of both patients and controls were born in Western Sicily. Controls were in good health according to their clinical history and blood tests (complete blood cell count, erythrocyte sedimentation rate, glucose, urea nitrogen, creatinine, electrolytes, C reactive protein, liver function tests, iron, and proteins) (see Table 1). Their demographic and clinical features, comorbidity conditions, and pharmacological treatments were collected (see Table 1). Furthermore,

Table 1: Demographic and clinical characteristics, comorbidity conditions, and pharmacological treatment of 161 patients affected by sporadic TAA, 128 control subjects, and 30 aorta controls.

Variables	Patients ($N = 161$)	Male ($N = 127$)	Female ($N = 34$)	Controls ($N = 128$)	$P1$ (cases versus controls)	$P2$ (male versus female)	Aorta Controls ($N = 30$)
Demographic characteristics							
Age, mean (SD)	63 (10.7)	63 (11)	64 (9)	61.1 (5.8)	0.834	0.594	63.9 (10.3)
Male sex, number (%)	127 (78)			61 (47)			
Female sex, number (%)	34 (22)			67 (53)			
Body mass index, mean (SD)	27 (4.3)	26.9 (3.8)	27.5 (5.6)	26.9 (2.9)	0.898	0.963	25.6 (2.9)
Size and location							
Size (mm), mean (SD)	53.3 (8)	52.9 (7.5)	55 (9.8)	0 (0)		0.191	0 (0)
Location, number (%):				0 (0)		0.198	
Ascending aorta	81 (50)	20 (59)	61 (48)				
Aortic Bulb	18 (11)	1 (3)	17 (13.4)				
Ascending aorta and Aortic bulb	62 (39)	13 (38)	49 (38.6)				
Comorbidity conditions, number (%)							
Cardiovascular Ischemic Familiarity	59 (36.6)	48 (38)	11 (32)	34 (27)	0.089	0.7	1 (3.3)
Smoking	73 (45)	67 (53)	6 (18)	66 (51)	0.351	**<0.001**	3 (10)
Hypertension	127 (78.9)	101 (80)	26 (76)	40 (31)	**<0.001**	0.879	2 (6.6)
Dislipidemy	37 (23)	30 (24)	7 (21)	20 (16)	0.158	0.886	0 (0)
Diabetes mellitus	24 (15)	16 (13)	8 (24)	16 (13)	0.677	0.187	0 (0)
Renal failure	5 (3.1)	4 (3.1)	1 (2.9)	0 (0)	0.168	0.621	0 (0)
Dissection	18 (11)	5 (15)	13 (10)	0 (0)		0.669	0 (0)
Aortic valve pathology, number (%):							0 (0)
Normal	90 (56)	71 (56)	20 (59)	0 (0)		0.766	0 (0)
Prolapse	21 (13)	17 (13)	3 (9)	0 (0)			0 (0)
Vascular calcium fibrosis	50 (31)	39 (31)	11 (32)	0 (0)			0 (0)
Aortic valve dysfunction, number (%):							
Normal	32 (20)	26 (20.4)	6 (17.6)	0 (0)		0.91	0 (0)
Faint incontinence	29 (18)	22 (17)	7 (20.4)	0 (0)			0 (0)
Moderate incontinence	34 (21)	28 (22)	6 (17.6)	0 (0)			0 (0)
Severe incontinence	44 (28)	35 (28)	9 (26.4)	0 (0)			0 (0)
Faint stenosis	1 (0.6)	1 (0.8)	0 (0)	0 (0)			0 (0)
Moderate stenosis	2 (1.2)	1 (0.8)	1 (3)	0 (0)			0 (0)
Severe stenosis	19 (11.2)	14 (11)	5 (15)	0 (0)			0 (0)
Atherosclerosis coronary syndrome No (%)	54 (33.8)	45 (36)	9 (26.5)	0 (0)		0.42	0 (0)
Drugs, number (%)							
Beta blockers	62 (39)	47 (37)	15 (44)	0 (0)			0 (0)
Central alpha-adrenergic agonists	26 (16)	21 (17)	5 (15)	0 (0)			0 (0)
Sartans	32 (20)	27 (21)	5 (15)	0 (0)			0 (0)
Calcium-channel blockers	47 (29)	38 (30)	9 (26)	0 (0)			0 (0)
ACE inhibitors	66 (41)	55 (43)	11 (32)	21 (16)			0 (0)
Antidiabetic drugs	19 (12)	13 (10)	6 (18)	16 (13)			0 (0)
Antiaggregant drugs	51 (32)	44 (34)	7 (21)	40 (31)			0 (0)
Antidislipidemic drugs	36 (22)	30 (24)	6 (18)	0 (0)			0 (0)
Diuretics	36 (22)	24 (19)	12 (36)	40 (31)			0 (0)

echocardiography imaging examinations confirmed absence of any aorta wall histopatological abnormalities in all controls.

In order to detect histopathological abnormalities, control ascending aortas were obtained from 30 individuals (20 men and 10 women, mean age: 63.9±10.3) who died for causes unrelated to aortic disease and having no sepsis at death time, as confirmed by autopsy (see Table 1).

Our study received approval from local ethic committees and all participants gave their informed consent. Data were encoded to ensure patient and control protection. All measurements were performed without knowledge about nature of material.

2.3. Histopathological Assays and Identification of MD Phenotypes. Histopathological investigations were performed only in 100 aorta samples obtained from aortic wall of patients who underwent surgical repair, since some patient's aortas showed unsuitable histological conditions. The procedures used were previously reported in our recent studies [16, 46–49] and briefly described in online Supplementary Material. In addition, the following histological features were evaluated: (1) fibrosis (defined as an increase in interstitial collagen); (2) medionecrosis (defined as a focal loss of smooth muscle cell nuclei in the media); (3) cystic medial necrosis (defined as mucoid material accumulation); (4) focal or plurifocal medial apoptosis; (5) elastic fragmentation (defined as focal fragmentation of elastic lamellae in the media); (6) amounts of MMP-9; (7) inflammatory/immune cell infiltration. Histopathological abnormalities of aortic wall were graded and defined according to the definitions and grading systems used by Bechtel and colleagues [50] (see details reported in the section of online Supplementary Materials and precisely in Figures 1(S) and 2(S).) and previously described in our recent studies [16, 46–49].

In addition, typical phenotypes were detected as reported in online Supplementary Material and previously described in our recent study [49].

2.4. Immunohistochemical Assays. Immunohistochemical analyses were performed on aorta sections. Specific monoclonal antibodies were used, and standard techniques were performed as described in online Supplementary Material.

2.5. Tunel Testing. For detecting apoptosis, a TdT- (Terminal deoxynucleotidyl Transferase-) mediated X-dUTP (deoxyuridine triphosphate nucleotides) nick end-labeling (TUNEL) reaction ("In situ cell death detection kit", Roche Diagnostics S.p.A, Milano, Italy) was used and performed as reported in detail in online Supplementary Material. Three apoptosis patterns were identified: absent, focal, and plurifocal medial apoptosis, as evidenced in Figure 2(S) in online Supplementary Material.

2.6. Semiquantitative Evaluation of MMP-9 by Immunohistochemical Assays. A semiquantitative evaluation of MMP-9 amount in aortic specimens was performed. Staining was classified as low, moderate, or high amount, as reported in Figure 2(S) in online Supplementary Material.

2.7. DNA Samples and Molecular Typing. DNA samples were obtained by blood samples from case and control individuals (see online Supplementary Material). They were genotyped for ten SNPs located in promoter and coding regions of selected candidate genes codifying molecules related to TLR4-mediated signaling pathway and with biological effects able to modulate the susceptibility for several CVDs, such as sporadic TAA. Information about these SNPs was acquired from dbSNP NCBI, the ENSEMBL database (http://www.ensembl.org/index.html), and the UCSC Genome Browser website (http://genome.ucsc.edu) and reported in Table S1 of online Supplementary Material. Genotyping was performed using the procedures illustrated in online Supplementary Material.

2.8. Inflammatory Plasma Molecule Measurements. Plasma IL-6, TNF-α, MMP-2, MMP-9, and CRP levels were measured according to method reported in online Supplementary Material.

2.9. Assessment of Mean Terminal Restriction Fragment Length. A marker of telomere leukocyte length was determined using DNA samples of 30 cases and 30 controls selected randomly, but having the same age and gender. The procedure used was previously described in the study of Balistreri and colleagues [51].

2.10. Statistical Analysis. All analyses were performed with R and Microsoft Excel software. Significant differences among qualitative variables were calculated by using Pearson χ^2 test. To analyze significant relationships among quantitative variables, Wilcoxon rank sum test was employed. Furthermore, odds ratios (OR) with 95% confidence intervals (CI) and their significance were calculated. To study mortality between male and female patients, Kaplan-Meier survival functions were calculated. Differences between survival curves with a Gehan test and appropriate Peto & Peto modification were carried out.

Allele and genotype frequencies were evaluated by gene count. Data were tested for goodness of fit between observed and expected genotype frequencies according to Hardy-Weinberg equilibrium, by χ^2 tests. Significant differences in frequencies among groups were calculated by using χ^2 test and appropriate tables (2×2 and 3×3 tables, etc., were appropriate and corrected by Bonferroni). Significant relationship between genetic variables and pathology risk was analysed using quasi-likely hood binomial models.

Analysis of variance (ANOVA) test (corrected by Bonferroni) was also utilised to compare positive inflammatory/immune cells between case and control aorta samples. Unpaired t-test (Welch corrected) was, utilised to analyse positive inflammatory/immune cells between pathological and normal case aortas. To identify possible correlations between CD3+CD4+CD8+CD68+ cell number and aorta aneurysm diameter, nonparametrical Spearman correlation test was also used. The same test was utilised to evaluate the correlations between the severity of all examined histopathological abnormalities and patient features or the major risk

factors (i.e., age, gender, aortic diameter, hypertension, diabetes, and smoking) and to assess the correlations between the MMP-9 and severity of elastic fragmentation.

Quantitative values of the cytokines, MMPs and CRP, were expressed as mean ± SD. To assess their differences, unpaired t-test (Welch corrected) was utilised. Categorical variables were compared by chi-square test or Fisher's exact test. Their correlations were assessed using Spearman's rank correlation. A $P < 0.05$ was considered statistically significant.

The difference in mean TRF length between cases and controls was analysed using an independent sample "t" test. The difference in mean TRF length in subjects with different risk factors was analysed using bivariate correlation for continuous variables and independent samples "t" test for categorical variable. The independent effect of age, sex, and other risk factors on the mean TRF length was analysed using a linear regression model controlling for case control status.

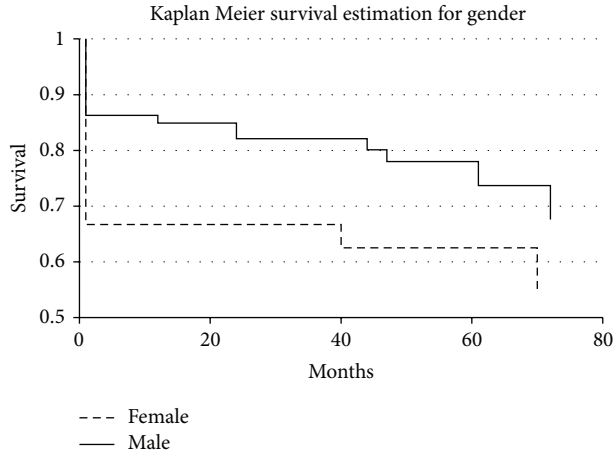

Figure 1: Survival in female and male patients after surgery.

3. Results

3.1. Patient and Control Characteristics. We analyzed the patient and control features summarized in Table 1. The elevated number of sporadic TAA men (127 versus 34 women) led us to compare all patient features according to gender. No statistical significant differences were detected, with exception of smoking (67 male versus 6 female, $P < 0.001$ by Pearson χ^2 test, 2 × 2 table; OR 5.16 (1.92–16.32), $P = 0.0002$ by Fisher test). However, among the major risk factors, hypertension characterised 79% of all patients, opportunely treated with medications like ACE inhibitors and beta-blocker, and so forth, during the follow-up and after surgery (see Table 1). According to gender, no significant differences were also observed in evaluating long-term survival (80 months) after surgery. However, OR was higher for women (4.5 (3.2–6.9) versus 2.6 (2.2–2.9) for men). Significant differences were, detected in short-term survival (30 days). Compared with male patients, females showed increased 30 day mortality: 9 of 34 female (26%) versus 10 of 127 male (8%) ($P = 0.049$ by Gehan test; OR 6.5 (1.5–6.8), $P = 0.01$ by Fisher test) (see Figure 1). These data correlated with several clinical conditions observed in the major female number after surgical repair (data not shown). Comparisons between patient and control features were also assessed. No statistical significant differences were detected, with exception of hypertension (127 versus 40 $P < 0.001$ by Pearson χ^2 test, 2 × 2 table; see Table 1).

3.2. Allele Frequencies of the Ten TLR4 Related Pathway SNPs and Identification of a Combined Risk Genotype Related to TLR4-Mediated Signaling Pathway. In order to demonstrate the key role of TLR4-mediated signaling pathway in the pathophysiology of sporadic TAA and to support our hypothesis, we compared the allele frequencies of ten selected SNPs between 161 patients and 128 controls. Significant differences were only found for the following SNPs: rs4986790 TLR4, rs333 CCR5, rs2070744 eNOs, rs1799752 ACE, rs3918242 MMP-9, and rs2285053 MMP-2 (see Table 2). Among these, we interestingly observed that the rs4986790 TLR4 polymorphism confers a higher susceptibility for sporadic TAA (OR = 14.4, $P = 0.0008$) (see Table 2). The protective +896 G TLR4 allele associated with a low risk of age-related diseases [32] has a frequency only of 0.3% (1) in cases versus 4% (11) in controls (see Table 2). In addition, using a quasi-hood binomial statistical model, we obtained that the rs4986790 TLR4, rs1799752 ACE, rs3918242 MMP-9, and rs2285053 MMP-2 SNPs are independent risk factors for sporadic TAA ($P = 0.001$). Considering the biological effects of these SNPs (see Table S1), we assessed the frequency of +896ATLR4/DACE/−1562TMMP-9/−735TMMP-2 risk genotype in cases and controls. By comparing it with frequency of other combinations, combined risk genotype, "*high responder genotype,*" was significantly represented in cases than controls. Indeed, 46 patients were carriers of the combined risk genotype versus 10 controls ($P = 0.000009$, by χ^2 test; OR = 4.7, $P < 0.0001$ by Fisher's exact test, see Table 3).

3.3. Evaluation of the Role of +896ATLR4/DACE/−1562TMMP-9/−735TMMP-2 Combined Risk Genotype in Influencing the Levels of Systemic Plasma Inflammatory Mediators. Given the overrepresentation of this combined risk genotype in patients and its strong role in the susceptibility (OR = 4.7, see Table 3) for sporadic TAA, we evaluated its biological effect in influencing the grade of chronic inflammation. Thus, we assessed the eventual significant differences in systemic plasma levels (AASSP levels) of IL-6, TNF-α, CRP, and MMP-2 and -9 between all patients and all controls. The same analysis was performed in cases bearing combined risk genotype versus no case carriers and in case carriers versus control carriers. As reported in Table 4, we detected significant differences of all systemic plasma mediators between patients and controls. However, the very interesting and promising datum was the presence of higher levels of all mediators examined in cases bearing combined risk genotype than both cases bearing other genotypes and control carriers of combined risk genotype, as illustrated clearly in Table 4. Furthermore, we detected

Table 2: Allele frequencies of rs4986790 (+896A/G) TLR4, rs333 (Δ32) CCR5 deletion, rs2070744 (−786T/C) eNOs, rs1799752 (D/I) ACE, rs3918242 (−1562C/T) MMP-9, and rs2285053 (−735C/T) MMP-2 SNPs in 161 S-TAA patients and 128 matched controls (2 × 2 comparisons between the different groups with odd ratio (OR) and 95% confidence interval).

Candidate genes	Reference SNP number	Alleles	Patients (N = 161)		Matched controls (N = 128)		P (2 × 2 tables)	OR (95% CI)
TLR4	rs4986790	+896A	321	99.7%	245	96%	0.0008	14.4 (18.1–112.4) P = 0.0008
		+896G	1	0.3%	11	4%		
CCR5	rs333	WT	317	98%	238	93%	0.001	4.7 (1.7–13.1) P = 0.001
eNOS	rs2070744	Δ32	5	2%	18	7%	0.00007	2.2 (1.5–3.2) P < 0.0001
		−786T	207	64%	204	80%		
		−786C	115	36%	52	20%		
ACE	rs1799752	I	125	39%	141	55%	0.0001	1.9 (1.3–2.6) P = 0.0001
		D	197	61%	115	45%		
MMP-9	rs3918242	−1562C	282	88%	241	94%	0.011	2.27 (1.2–4.22) P = 0.01
		−1562T	40	12%	15	6%		
MMP-2	rs2285053	−735C	287	89%	251	98%	0.00005	6.1 (2.3–15.8) P < 0.0001
		−735T	35	11%	5	2%		

All genotypes were in Hardy-Weinberg equilibrium.

Table 3: Frequency of +896ATLR4/DACE/−1562TMMP-9/−735TMMP-2 "high responder" (proinflammatory) genotype between patients and controls (2 × 2 comparisons between the different groups with odd ratio (OR) and 95% confidence interval).

Subjects	+896ATLR4/DACE/−1562TMMP-9/−735TMMP-2 "high responder"	Other genotypes	P (2 × 2 Table)	OR (95% CI)
Patients (N = 161)	46	115	**P = 0.000009**	**4.7 (2.7–9.8)** **P < 0.0001**
Controls (N = 128)	**10**	**118**		

Table 4: Systemic plasma mediator's levels "AASSP" from patients and controls.

Systemic mediators examined	Patients (N = 161)	Controls (N = 128)	P values*
IL-6 (pg/mL)	13.69 ± 2.1	5.1 ± 1.9	<0.0001
TNF-α (pg/mL)	16.34 ± 1.2	8.1 ± 2.4	<0.0001
CRP (mg/L)	16.86 ± 2.2	5.6 ± 1.3	<0.0001
MMP-2 (ng/mL)	57.5 ± 2.8	13.54 ± 1.24	<0.0001
MMP-9 (ng/mL)	59.8 ± 2.5	12.7 ± 1.6	<0.0001
Systemic mediators examined	Patients with high responder genotype (N = 46)	Patients with other genotypes (N = 115)	P values*
IL-6 (pg/mL)	17.66 ± 2.1	9.1 ± 0.9	<0.001
TNF-α (pg/mL)	16.78 ± 1.2	10.1 ± 2.2	<0.01
CRP (mg/L)	20.13 ± 1.7	12.1 ± 0.5	0.01
MMP-2 (ng/mL)	61.8 ± 3.8	26.54 ± 1.6	<0.0001
MMP-9 (ng/mL)	59.7 ± 3.7	21.7 ± 2.6	<0.0001
Systemic mediators examined	Patients with high responder genotype (N = 46)	Controls with high responder genotype (N = 10)	P values*
IL-6 (pg/mL)	17.66 ± 2.1	8.66 ± 2.1	<0.0001
TNF-α (pg/mL)	16.78 ± 1.2	10.78 ± 1.2	<0.01
CRP (mg/L)	20.13 ± 1.7	6.13 ± 1.7	<0.0001
MMP-2 (ng/mL)	61.8 ± 3.8	18.8 ± 3.9	<0.0001
MMP-9 (ng/mL)	59.7 ± 3.7	12.7 ± 2.7	<0.0001

*By unpaired t-test with Welch correction.

Table 5: Comparison of systemic inflammatory mediator's levels "AASSP" from controls bearing combined risk genotype versus controls with other genotypes and between controls bearing +896A TLR4 allele versus controls with +896G TLR4 allele.

Systemic mediators examined	Controls with combined risk genotype ($N = 10$)	Controls with other genotypes ($N = 118$)	P values*
IL-6 (pg/mL)	8.66 ± 2.1	1.1 ± 0.69	<0.0001
TNF-α (pg/mL)	10.78 ± 1.2	2.1 ± 1.2	<0.0001
CRP (mg/L)	6.13 ± 1.7	0.9 ± 1.5	<0.0001
MMP-2 (ng/mL)	18.8 ± 3.9	2.54 ± 1.3	<0.0001
MMP-9 (ng/mL)	12.7 ± 2.7	1.7 ± 1.6	<0.0001
Systemic mediators examined	Controls with +896ATLR4 allele ($N = 10$)	Controls with +896G TLR4 allele ($N = 118$)	P values*
IL-6 (pg/mL)	5.1 ± 0.9	0.9 ± 0.5	<0.0001
TNF-α (pg/mL)	7.1 ± 0.6	2.1 ± 1.2	<0.001
CRP (mg/L)	4.3 ± 1.1	0.3 ± 1.9	<0.0001
MMP-2 (ng/mL)	10.6 ± 1.8	1.8 ± 0.9	<0.0001
MMP-9 (ng/mL)	8.3 ± 0.7	0.98 ± 1.5	<0.0001

*By unpaired t-test with Welch correction.

that higher plasma levels of MMP-2 and -9 levels from cases bearing combined risk genotype significantly correlated with the moderate and elevated amounts of MMP-9 detected reciprocally from their tissue aorta samples ($r = 0.397$, $P = 0.001$; $r = 0.234$, $P = 0.03$ by nonparametrical Spearman correlation test; data not shown). In particular, cases having combined risk genotype showed a significant association between increased plasma MMP-9 and MMP-2 levels and elevated amounts of MMP-9 ($P = 0.006$ by χ^2 test; data not shown). Consistent with these data, positive correlations were detected in the cases with combined risk genotype between the increased plasma levels of MMP-9 and MMP-2 and elastic fragmentation and the elevated amounts of MMP-9 observed in their tissue aorta samples ($r = 0.497$, $P = 0.0001$; $r = 0.267$, $P = 0.03$, $r = 0.342$, $P = 0.006$, resp., by nonparametrical Spearman correlation test; data not shown).

3.4. Assessment of the Biological Effect of +896ATLR4/DACE/−1562TMMP-9/−735TMMP-2 Combined Risk Genotype, "High Responder Genotype," and Alleles of TLR4 Gene in the Healthy Control Group. In verifying the influence and the biological effect of combined risk genotype on levels of systemic inflammatory mediators, and consequently its role in the occurrence of VR, MD and their complications, such as sporadic TAA, their quantities were compared in control carriers versus no control carriers (10 versus 118, as reported in Table 3). As shown in Table 5, this comparison demonstrated significant differences of all systemic inflammatory mediators (IL-6, TNF-α, CRP, and MMP-2 and -9) in controls bearing the high responder genotype than those with other genotypes. Thus, combined risk genotype seems to mediate a crucial biological effect on the levels of systemic inflammatory mediators and, hence, in the evocation of MD, VR, and sporadic TAA.

We also evaluated the potential significant differences of levels of systemic AASSP mediators in controls screened only for the presence of rs4986790 TLR4 polymorphism. Controls bearing the allele +896G, associated with a blunted innate/inflammatory response, showed significant reduced levels of all systemic AASSP mediators than carriers of the +896A TLR4 allele (see Table 5). Thus, controls with the TLR4 proinflammatory +896A allele had significant levels of systemic mediators, but their magnitude was lower than that observed in controls with the combined risk genotype.

3.5. Detection of the Inflammatory Cell Infiltration in Tissue Aorta Samples from Patients and Controls and the Examination of the Role Mediated of the Combined Risk Genotype. Furthermore, infiltration of lymphocytes and macrophages was also detected in tissue aorta wall samples from patients, control aortas, and normal areas from the same TAA tissues. A significant higher infiltrate of lymphocytes and macrophages in tissue aorta wall samples from patients compared with both control aortas and normal areas from the same TAA tissues was observed (see Figure 2). Interestingly, the infiltration of inflammatory/immune cells was particularly considerable in the vasa vasorum of adventitia from the aorta patient samples. In contrast, a very small infiltrate of these cells was observed in control aortas, which appears to be less significant in respect to that found in normal aorta TAA areas. CD20+ cell infiltrate was less represented in three groups, even if significant differences were observed by comparing the three cohort tissues (see Figure 2). Immunostaining with CD68 antibody also indicated that macrophages were prevalently present in aortas from TAA patients in respect to control aortas and normal areas from the same TAA tissues (see Figure 2).

In order to validate the biological effect mediated of combined risk genotype on the levels of systemic plasma inflammatory mediators, we also compared inflammatory/immune infiltrate between patients bearing high responder genotype and those with other genotypes. According to encouraging data obtained on levels of systemic plasma inflammatory mediators, we assessed a higher inflammatory/immune infiltrate in tissue aorta samples from patients bearing high

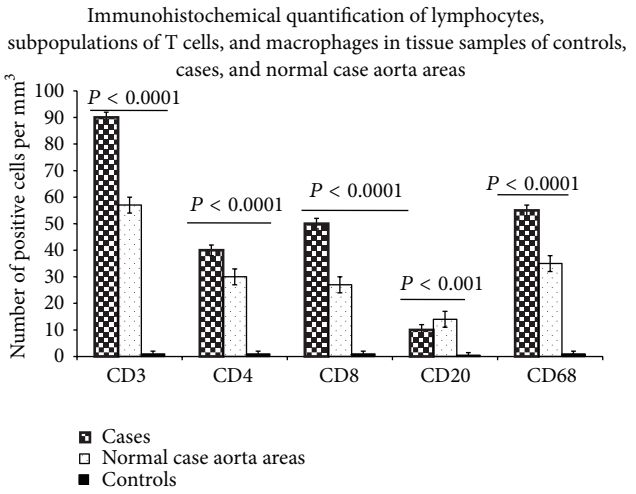

Figure 2: Morphometric quantification of lymphocytes, T cell subpopulations, and macrophages in tissue samples of the control aortas and patients and normal aorta case areas. CD3, CD4, CD8, CD20, and CD68 positive cells in media and adventitia and in 10 contiguous high-power fields (magnification 400x) were counted by two independent observers. Significant increased amounts of CD3+CD4+CD8+CD68+CD20+ cells were observed by comparing their values among the three groups (by ANOVA test). In particular, cases showed significant higher numbers of these cells than controls and normal aorta case areas.

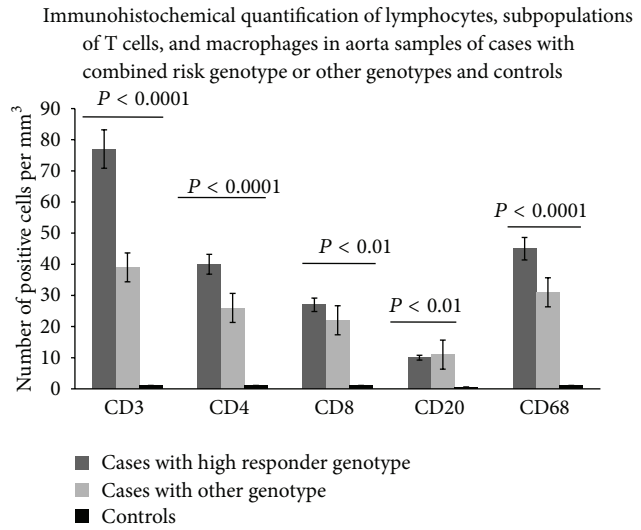

Figure 3: Morphometric quantification of lymphocytes, T cell subpopulations, and macrophages in aorta samples of cases with high responder genotype, other genotypes, and controls. CD3, CD4, CD8, CD20, and CD68 positive cells in media and adventitia and in 10 contiguous high-power fields (magnification 400x) were counted by two independent observers. Significant increased amounts of CD3+CD4+CD8+CD68+CD20+ cells were observed by comparing their values among the three groups (by ANOVA test). In particular, cases with high responder genotype had higher numbers of these cells than controls and cases with other genotypes.

responder genotype than those bearing other genotypes and control aortas (see Figure 3). Positive correlation was identified between the number of CD3+CD4+CD8+ CD68+ cells observed in aorta samples from patients bearing high responder genotype and the histological abnormalities observed through histopathological and immunohistochemical assays and Tunel testing (see Table 6). As reported in Table 6, the number of CD3+CD4+CD8+ CD68+ cells also correlated with the increased plasma levels of IL-6, TNF-α, CRP, and MMP-2 and -9.

3.6. Identification of Typical MD Phenotypes and Their Potential Association with Combined Risk Genotype. In addition, the 46 patients with combined risk genotype showed for the 89% a typical morphological aorta's phenotype, defined in our previous study as phenotype III, characterized by elevated cystic MD, plurifocal medial apoptosis, and increased MMP-9 amount (as reported in online Supplementary Material) [42]. Positive correlations were identified between the severity of histopatological abnormalities characterising this phenotype and hypertension, smoking, and age ($r = 0.179$, $P = 0.03$; $r = 0.345$, $P = 0.001$; $r = 0.267$, $P = 0.02$, resp., by nonparametrical Spearman correlation test; data not shown).

3.7. Assessment of the Weight of Combined Risk Genotype in Inducing Vascular Biological Ageing: Analysis of the Gold Marker of Biological Ageing "Telomere Attrition". Consistent with these interesting data, we also evaluated whether the combined risk genotype can likely have a strong role in determining vascular senescence and onset of sporadic TAA.

Table 6: Correlations between the number of CD3+CD4+CD8+ CD68+CD20+ cells observed in aorta samples from patients bearing combined risk genotype and the histological abnormalities observed through histopathological and immunohistochemical assays and Tunel testing and levels of IL-6, TNF-α, CRP, and MMP-2 and -9.

Variables	Correlations	P values*
Medionecrosis of grade III	0.278	0.02
Cystic-medial change of grade III	0.346	0.001
Elastic fragmentation of grade III	0.467	0.0001
Plurifocal Medial apoptosis	0.333	0.001
Elevated MMP-9 amounts	0.379	0.001
IL-6	0.379	0.003
TNF-α	0.445	0.001
CRP	0.467	0.002
MMP-2	0.578	0.001
MMP-9	0.502	0.001

*By nonparametrical Spearman correlation test.

To this purpose, we examined the mean of blood leukocyte telomere length using terminal restriction fragment assay (TRF test, a southern blot technique) and blood samples from 30 patients and 30 controls, using a procedure described in two of our recent studies [51]. Thus, we detected that the case group had a mean TRF length (4.675 ± 0.605 kbp, data not shown), significantly lower than that observed in the control group (6.218 ± 0.485 kbp, data not shown). A difference of 1.543 bp was observed between cases and controls (95%

confidence interval 68 bp to 201 bp, $P = 0.001$). There was no significant change in the mean TRF length with age in both patients and controls (6 bp decrease per year, SD = 4; $P = 0.25$). There was also no significant correlation between aneurysm size and mean TRF length ($P = 0.46$). There was no significant difference in the mean TRF length between male and female patients. Comparisons between mean TRF length and the risk TAA factors including gender, smoking, hypertension, diabetes, and family history were also evaluated. Sex as an independent risk factor did not have any significant effect on the mean TRF length ($P = 0.76$). In contrast, smoking and hypertension in S-TAA cases were the only risk factors which were significantly associated with the mean TRF length ($P = 0.01$). S-TAA subjects with smoking and hypertension history had a significantly shorter mean TRF length (4.491, SD = 0.237) compared to subjects without these risk factors (5.997, SD = 0.302), (mean difference 132 bp, confidence interval 11 bp to 245 bp, $P = 0.01$).

Stratifying the 30 patients having low mean TRF length for combined risk genotype, we interestingly observed that 85% (versus 8% for controls) were carriers of combined risk genotype ($P = 0.001$ by χ^2 test, 2×2 table; data not shown) and had significant higher levels of plasma inflammatory mediators (IL-6: 13.8 ± 2.3 versus 5.1 ± 1.4, $P < 0.001$; TNF-α: 15.5 ± 1.4 versus 8.2 ± 1.2, $P < 0.001$; CRP: 18.4 ± 1.3 versus 6.6 ± 2.8, $P < 0.0001$; MMP-9: 59.9 ± 2.8 versus 11.6 ± 1.8, $P < 0.0001$, and MMP-2: 57.8 ± 3.5 versus 13.7 ± 1.9, $P < 0.0001$, resp.; data not shown), increased amounts of CD3+CD4+CD8+CD68+CD20+ cells than controls and patients with other genotypes ($P < 0.0001$, by ANOVA test (corrected by Bonferroni)).

4. Discussion

Sporadic TAA is predominantly a silent ailment, until rupture or dissection occur, and insidious in its onset and progression. Its diagnosis is exclusively based on imaging technologies (i.e., ECHO, CT, or MRI) [12]. Accordingly, it is very crucial to early predict, diagnose, and treat sporadic TAA, characterised by lack of medical optional treatments and disease biomarkers, such as blood tests. Blood tests might consent to detect in general population individuals at sporadic TAA risk, to monitor its progression and predict complications. To this purpose, it should be crucial understanding cellular and molecular mechanisms and genetic risk factors. Recently, it has been suggested that aortic aneurysms, and particularly the sporadic forms, are immune diseases with a strong genetic component [13]. In particular, a particular involvement of chronic inflammation is emerging. We have evidenced this crucial aspect of the complex pathophysiology of this disease in our recent studies [16, 46–49]. In patients with TAAD, we observed high levels of immune/inflammatory cells and a significant association of some inflammatory polymorphisms with the TAAD susceptibility. In line with this, He and colleagues recently observed an increased immune/inflammatory infiltrate in aorta samples of patients with sporadic TAA [14, 15]. This is leading to identify the inflammatory pathways, which might operate as key link between the onset of sporadic TAA and immune system. Their recognition should be very imperative in order to translate experimental data in clinical new personalized measures of TAA prevention, diagnosis, treatments, and management.

Considerable and convincing evidence links the pathophysiology of atherosclerosis, cardiac dysfunction, congestive heart failure, and other vascular diseases with the TLR-4-mediated signaling pathway, as amply stressed by Frantz and colleagues [31]. Recently, the group of Pasterkamp also provided an overview of the endogenous molecules, released under cellular cardiovascular stress and damage, which can trigger innate immunity via TLR-4-mediated signaling pathway in CVDs [33]. In the specific case of sporadic TAA, recent experimental investigations in animal and *ex vivo* models also emphasise its role in the vascular aorta alterations (VR and MD) and their complications, such as sporadic TAA, by evocating or modulating increased expression and activation of endothelium dysfunction and remodeling aorta pathways [23, 37–42]. On the other hand, Pryshchep and colleagues demonstrated the TLR4-mediated signaling pathway expression in all cells of arterial wall and particularly in ECs and VSMCs. In addition, they also evidenced its functional importance in both mediating physiological aorta homeostasis and maintaining protection, as well as in inducting pathological aorta phenotypes, that is, VR and MD [23]. Furthermore, Song and colleagues demonstrated that signaling via TLR4-mediated signaling pathway and its signal adaptors, that is, MyD88, is responsible for the age-elevated basal IL-6 response using VSMCs from aged $TLR4^{-/-}$ and $Myd88^{-/-}$ mice [37]. Eissler and colleagues observed an increased hypertension-related expression of TLR4-mediated signalling pathway in vascular cells of untreated hypertensive rats [38]. The group of Golzales-Ramos underlined that circulating Heat Shock protein 70, associated with an increased cellular aorta's damage, regulates the profibrotic response of human aorta SMCs through increased transforming growth factor type-1 (TGF-1) expression, evocated by TLR4-mediated signaling pathway [39]. In addition, Li and colleagues reported the role of TLR4-mediated signaling pathway in regulating the MMP-9 expression in human VSMCs [40]. Bucci and colleagues recently emphasized as the vascular thoracic aorta homeostasis and its alteration in rats is based on the activity of TLR4-mediated signaling pathway and its cross talk with other stress and stretch pathways, that is, ACE, eNOs, and MMP pathways [41]. Furthermore, a recent study demonstrated in apolipoprotein E-deficient mice that it is possible to limit the inflammatory process by blocking TLR4/c-Jun N terminal kinase signaling pathway with Rosiglitazone in the initiation stages of aortic aneurysm development [42].

This encouraging and increasing evidence, fruit prevalently of animal investigations, and our recent data on TAAD [16, 46, 48], led us to analyze the potential role of genetic variants related to TLR4-mediated signaling pathway in the complex pathophysiology of sporadic TAA. Until now, no literature data exist about their role in sporadic TAA. Thus, our study represents the first report which, through a human *ex vivo* study approach, evidenced as some polymorphisms related to TLR4-mediated signaling pathway significantly

modulate the sporadic TAA risk: rs4986790 TLR4, rs333 CCR5, rs2070744 eNOs, rs1799752 ACE, rs3918242 MMP-9, and rs2285053 MMP-2 polymorphisms. Among these, the rs4986790 (+896A>G) TLR4 polymorphism confers a higher susceptibility for sporadic TAA (OR = 14.4, $P = 0.0008$). However, it represents an independent risk TAA factor for sporadic TAA, as well as the rs1799752 ACE, rs3918242 MMP-9, and rs2285053 MMP-2 SNPs. Thus, we assessed whether their combined genotype constitutes a risk profile. A significant overrepresentation of their combined risk genotype (+896ATLR4/DACE/-1562TMMP-9/-735TMMP-2) was observed in cases than controls (46 versus 10, $P < 0.000009$) by comparing it with frequency of other combinations. In addition, it was associated a significant risk for sporadic TAA (OR = 4.7; $P < 0.0001$). Cases bearing combined risk genotype showed higher systemic inflammatory mediator levels than those with other genotypes and control carriers. In particular, they had higher plasma levels of MMP-9 and -2 which correlated with the amounts of MMP-9 and elastic fragmentation observed in their tissue aorta samples. A higher chronic inflammatory infiltrate was also found in cases bearing combined risk genotype, which positively correlated with histological abnormalities and levels of mediators. In addition, they showed in their tissue aorta samples a typical morphological phenotype, characterized by elevated cystic MD, plurifocal medial apoptosis, and increased MMP-9 amounts, and defined in a previous study as phenotype III [42]. Furthermore, we detected that combined risk genotype influences vascular biological ageing, evaluating the gold standard ageing marker, the telomere length, in a small number of cases and controls, selected randomly, but having the same age and gender. It characterized the 85% of the cases examined, which had lower telomere length, higher levels of mediators, increased amount of chronic inflammatory infiltrate. This results concord with the preliminary data reported in a previous study and the recent literature reports [51–53].

These interesting results led us to evaluate the biological effect of the combined risk genotype. Thus, we analysed the levels of systemic mediators in healthy group. Controls bearing high responder genotype showed higher levels of systemic mediators than control carriers of only the rs4986790 TLR4 polymorphism. These results are in agreement with our previous data [54, 55]. Indeed, in clarifying and confirming the biological effects of rs4986790 TLR4 polymorphism and its role in the pathophysiology of age-related diseases, including CVDs, Alzheimer disease, prostate cancer, diabetes, and longevity, we assessed the levels of IL-6, TNF-α, IL-10, and eicosanoids in LPS-stimulated whole blood samples *in vitro* of 50 young healthy Sicilians, screened for the presence of rs4986790 TLR4 and −765G>C PTGS2, −1708G>A 5-Lo polymorphisms [54]. Significantly higher levels of both proinflammatory cytokines and eicosanoids were observed in individuals bearing the rs4986790 TLR4 polymorphism. However, their magnitude significantly was more increased in individual's carriers of their combined genotype. In addition, significantly lower levels of inflammatory mediators were observed in carriers bearing the TLR4 mutation (the +896G allele), whereas the anti-inflammatory IL-10 values were higher [54]. The same results were detected in this study in the controls carriers of the +896G TLR4 allele. Thus, these data strengthen our suggestion, stressed for other age-related diseases including Alzheimer disease and prostate cancer, that polymorphisms related to TLR4-mediated signaling pathway may have a major influence on the pathophysiology of a disease, such as sporadic TAA, when they operate in combination to create a "risk profile" [56, 57].

5. Limitations and Conclusions

In the complex, our data seem to suggest the strong relevance of TLR4-mediated signaling pathway in inducing MD, age related VR, and their complications, such as sporadic TAA. On the other hand, our findings emphasise as a combined risk genotype associated of TLR4-mediated signaling pathway are able to modulate the grade of aorta age-related phenotypical, histological, and systemic abnormalities and consequently vascular aorta ageing, onset, and progression of sporadic TAA. They also lead us to suggest that this signaling pathway might also be an optimal target for new therapeutic treatments able to retard or block the typical aorta age-related changes which determine endothelial dysfunction, MD, and VR. This might open new perspectives for the prevention of both aortic VR and MD and sporadic TAA, by using combined risk genotype (+896ATLR4/DACE/−1562TMMP-9/−735TMMP-2) as optimal genetic biomarker for the earlier detection of this silent pathology in preliminary phases and to treat with different and specific therapies depending on individual's genotypes. Consequently, it also leads to view vascular ageing as a modifiable risk factor, particularly for aortic disease. In the specific case of sporadic TAA, the therapeutic potential of TLR4-mediated signaling pathway might be defined through the use of its agonists or antagonists, whose effects have been prevalently experimented in mice, rats, and cultures as reported in literature [35]. This might consent to reduce chronic age-related inflammation and limit the dysfunction of ECs and endothelial progenitor cells (EPCs), which move towards injured endothelium or inflamed tissues and incorporate into foci of neovascularisation, thereby improving blood flow and tissue repair [58–60]. On the other hand, a recent investigation reports the involvement of TLR4-mediated signaling pathway in maintaining the stem cell phenotype of EPCs and enlarging this population [61]. This finding reveals a novel aspect of the multiple-faced TLR-4-mediated signaling pathway biology, and it may open new prospects for using TLR4 agonists in promoting the production of EPCs for clinical use. In addition, recent research underlines that miRNAs might play important roles in this scenario, modulating TLR-4-mediated signaling pathway activation [62]. MiRNAs seem, indeed, to have two opposite roles: TLR-4-mediated pathway activation and NF-κB signaling inhibition, in a complex scenario where low and chronic inflammation prevails, likely also sustained by cell senescence secretome. MiRNAs inhibition effect probably belongs to the different levels of anti-inflammatory pathways which have evolved to abate TLR-4 signaling to prevent cell and aorta destruction [62].

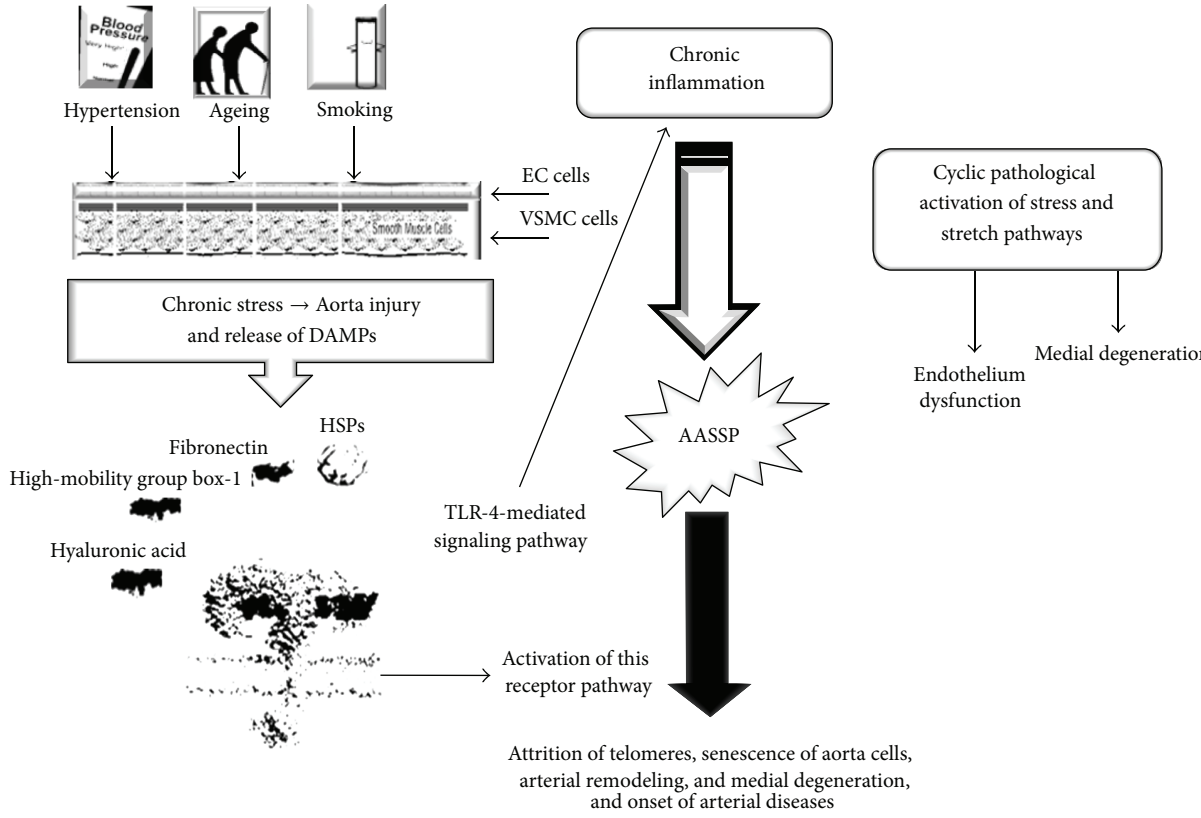

Figure 4: Our model about the pathophysiology of sporadic TAA, the *model of the pathway from the double-face*. The major risk factors, hypertension, age, and smoking, induce an increased production of reactive oxygen species (ROS) [17–19] and an upregulation of local renin-angiotensin system [16] and the tissue injury, initially involving ECs and subsequently VSMCs. This determines the release of some damage-related products and proteins (i.e., heart shock proteins (HSPs), high-mobility group box-1, low molecular hyaluronic acid, fibronectin fragments, and others), called danger-associated molecular patterns (DAMPs) [20]. DAMPs alert innate/inflammatory immune system interacting with TLR4 mediated signaling pathway, able to recognize both pathogens and endogenous ligands [21, 22]. Originally described as part of the first-line defense against Gram-negative bacteria, the best known member of TLRs, the TLR4, expressed on leukocytes and a large array of tissue and cell types, such as all aortic wall cells (particularly ECs and VSMCs), responds to these signals [23]. As a consequence, TLR4 activates and triggers an inflammatory response [24–28]. In turn, this determines a typical phenotypic switching of EC and VSMC cells due to activation of stress and stretch pathways accompanied by their dysfunction and senescence [27, 28]. In particular, it implies a differential change in their gene expression profile due prevalently of activation of Nuclear Factor-κB (NF-κB) transcription factor and followed by production and release of the so-called *arterial-associated senescence secretor phenotype* (AASSP) characterized by numerous inflammatory mediators, mitotic and trophic factors, proteoglycans and metalloproteinases (MMP)s, such as MMP-2 and -9, and vasoactive molecules [17, 24–29]. In addition, this also induces the reduction of nitric oxide (NO) [28]. This complex scenario results in modifications of vascular tone and permeability and degradation of components of extracellular matrix (ECM) and elastic fragmentation. VR and MD are, hence, evocated, which can evolve in aneurysm, dissection and rupture of aorta wall [17].

Based on our findings, we also suggest another possible therapeutic intervention to apply in the preclinical phase in subject's carriers of combined high risk genotype to the aim to retard to limit the onset and progression of the vascular ageing and its complications, such as sporadic TAA. Precisely, we propose antibody-mediated stimulation of TAM receptors involved in the inhibition of the inflammatory response [29]. The sequential induction of this pathway and its integration with upstream TLR and cytokine signaling networks may impact the evocation of the release of inflammatory mediators limiting the inflammatory response and consent to modulate the telomere/telomerase system reducing the senescence of both the aorta wall cells and the EPCs able in repairing aorta injury [29]. On the other hand, patients with CVDs exhibit a reduced EPC number and function [63–65]. It has become increasingly apparent that these changes may be effected in response to enhanced oxidative stress, possibly as a result of systemic and localized inflammatory responses. Recent studies suggest that inflammation and oxidative stress modulate EPC bioactivity [63–65].

The weight of our findings and suggestions might be certainly implemented validating them in a larger sample size, even if our data are the result of a relatively small sample and a very homogenous population. In addition, gene expression analyses, immunohistochemical TLR4 quantification, and soluble TLR4 level detection represent further objectives of our future studies. They should consent to translate with

major emphasis our promising data in personalized treatments of a pathology, the sporadic TAA, which clinically and predominantly is silent, until rupture or dissection occurs, and insidious in its onset and progression. Furthermore, until now its diagnosis is also exclusively based on imaging technologies.

Finally, our obtained data led us to postulate a potential model about the pathophysiology of sporadic TAA, which might be defined as *model of the signaling pathway from the double-face,* given its features (see Figure 4). We foretell that it can lead several researchers to perform investigations focused to clear the complex puzzle of this pathology.

Nonstandard Abbreviations and Acronyms

AASSP: Arterial-associated senescence secretor phenotype
CT: Computerized tomography
ECHO: Echocardiography
MRI: Magnetic resonance imaging
SNPs: Single nucleotide polymorphisms
TAAD: Stanford type A aortic dissection
TRF: Terminal restriction fragment.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Giovanni Ruvolo and Carmela R. Balistreri contributed equally to this study. Dr. Balistreri and Professor Ruvolo were involved in conception and study design. Professor Ruvolo and Dr. Candore were involved in the support of study materials/patients. Dr. Pisano collected and assembled the clinical data of study population. Dr. Balistreri performed the major number of experimental assays. Professor Maresi was involved in histopatological and immunohistochemical assessments. Dr. Balistreri acquired the results obtained and performed their analysis in collaboration with Dr. Torretta. Dr. Balistreri and Professor Ruvolo were involved in the data interpretation and their translation in clinical suggestions. Dr Balistreri was involved in drafting the paper. Dr. Balistreri and Professor Ruvolo contributed in the critical revision of the text of paper. Dr. Balistreri and Professor Ruvolo contributed in the study supervision. Dr. Balistreri gave the final approval of the version to be published. All authors participated in the study, and they read and approved the final paper.

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Research Article

Early Effects of a Hypocaloric, Mediterranean Diet on Laboratory Parameters in Obese Individuals

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Calorie restriction is a common strategy for weight loss in obese individuals. However, little is known about the impact of moderate hypocaloric diets on obesity-related laboratory parameters in a short-term period. Aim of this study was to evaluate the variation of laboratory biomarkers in obese individuals following a Mediterranean, hypocaloric (1400–1600 Kcal/die) diet. 23 obese, pharmacologically untreated patients were enrolled and subjected to the determination of anthropometric variables and blood collection at baseline, 1 and 4 months after diet initiation. After 4 months of calorie restriction, we observed a significant decrease in body weight and BMI (both $P < 0.0001$), insulin ($P = 0.037$), HOMA-IR ($P = 0.026$), leptin ($P = 0.008$), and LDH ($P = 0.023$) and an increase in EGF ($P = 0.013$). All these parameters, except LDH, varied significantly already at 1 month after diet initiation. Also, lower levels of insulin ($P = 0.025$), leptin ($P = 0.023$), and EGF ($P = 0.035$) were associated with a greater (>5%) weight loss. Collectively, our data support a precocious improvement of insulin and leptin sensitivity after a modest calorie restriction and weight reduction. Moreover, EGF and LDH may represent novel markers of obesity, which deserve further investigations.

1. Introduction

Obesity, a chronic disorder whose prevalence is increasing in adults, adolescents, and children, is now considered a worldwide epidemic and an important issue in the health care [1, 2]. In many populations, the prevalence of overweight and obesity has rapidly increased in the past 20 years. In the USA, more than 35% of adults and almost 17% of young people were classified as obese in 2009-2010 [3], and the lifetime risk of developing overweight or obesity was estimated to be roughly 50 and 25%, respectively [4].

Obesity predisposes to a number of pathological conditions, including cardiovascular diseases [5, 6], type 2 diabetes [7, 8], metabolic syndrome [9], nonalcoholic fatty liver disease [10], certain types of cancer [11, 12], obstructive sleep apnea [13], osteoarthritis [14], and asthma [15], and increases the risk of premature death [16], leading to elevated health care costs worldwide [17].

Factors predisposing to obesity include excessive food intake, lack of physical activity, and genetic susceptibility [18]. However, obesity is a heterogeneous condition, and intra-abdominal accumulation of fat tissue has been shown to have a higher impact on the development of cardiometabolic risk factors and related disorders than total excessive adiposity [19]. Increased free fatty acid availability, the release of proinflammatory cytokines and adipokines from adipose tissue, hepatic insulin resistance and inflammation, and the consequent dyslipidemia are among the many metabolic disorders associated with this condition. Hormonal status and drug treatments may also play an important role [20].

Also, increased levels of some hemostatic factors have been described that may explain the prothrombotic risk of obese patients [21].

Although surgical [22] and pharmacological therapies [23] have been shown to be useful, hypocaloric diet still represents the main and essential step in the treatment of obesity, together with increased physical activity and changes in lifestyle [24]. Weight loss, in fact, is an important goal and has proved to be beneficial in preventing health risks related to obesity [25, 26]. In this context, many hypocaloric diets are available that can be potentially recommended, including Mediterranean diet [27].

The association between the effects of a low-calorie diet and obesity is however very complex and not yet fully understood. In particular, little is known about the pathogenetic mechanisms and their putative markers that link potential benefits of weight loss, in a short-term period, to the asymptomatic, obese patients.

To elucidate possible relationships between obesity, diet, and weight loss, herein, we evaluated the variation, at short time intervals, of some anthropometric and laboratory parameters in obese individuals, subjected to a Mediterranean, low-calorie diet. In the effort to identify useful precocious markers in the follow-up of these patients, we analysed laboratory parameters correlated with the pathophysiological changes linked to obesity, including indexes of insulin resistance, proinflammatory and prothrombotic markers, adipocytokines, and lipid profile.

2. Materials and Methods

2.1. Patients and Study Design. We designed a prospective, descriptive study in the routine management of patients with obesity.

52 patients that attended the Clinical Nutrition Operative Unit of our University and were not taking any medications were initially enrolled from January 2012 to May 2013. Due to the high drop-off rate, 23 patients (17 females and 6 males), age 52 (46–61 yrs) and average body mass index (BMI) 37 kg/m^2, completed the study. Obesity was classified, according to the WHO guidelines, in grade I (BMI: 30–34.9 kg/m^2), grade II (BMI: 35–39.9 kg/m^2), and severe obesity (BMI: ≥40 kg/m^2). Percentage of fat mass and percentage of lean mass were established at the enrollment by bioelectrical impedance analysis using the application Bodygram Pro (Akern, Pontassieve, FI, Italy). Any pharmacological treatment or intercurrent diseases during follow-up were assumed as exclusion criteria. Informed consent was obtained by each patient enrolled in the study.

After careful consideration of global nutrition, a personalized, Mediterranean hypocaloric diet (1400–1600 kcal/die) was prescribed, aiming at the achievement of a moderate weight loss. The proposed food plan included carbohydrates (55% of total calories), proteins (20% of total calories), and mostly mono- and polyunsaturated fats (25% of total calories). The intake of cholesterol in the diet accounted for less than 300 mg/day, while fibers were equal to 25–30 g/day. The diet was also balanced in terms of micronutrients intake.

Patients underwent two sessions of behavioral dietary counseling: one at the first visit, which focused on notions of nutrition, dietary errors, suggestions on food preparation techniques, and the importance of associating physical exercise (30 minutes of speed walking, 3 days/week) with diet therapy, and a second session at 1 month from dietary treatment, to reinforce adherence to diet. During the first visit, patients were also subjected to anthropometric determinations (body weight and height). During the first visit, patients were also subjected to anthropometric determinations, including body weight and height for BMI calculation, and blood collection. Fasting blood samples were collected from all participants with no caloric intake for at least 12 h, and the following biochemical analyses were performed: glucose, insulin, cystatin C, adipokines (leptin, adiponectin, resistin, and visfatin), prothrombin time (PT), activated partial thromboplastin time (aPTT), fibrinogen, plasminogen activator inhibitor-1 (PAI-1), triglycerides, total cholesterol, and HDL cholesterol, γ-glutamyltransferase (γGT), aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), high-sensitivity C reactive protein (hsCRP), tumor necrosis factor α (TNFα), interleukins (IL): IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, and IL-10, monocyte chemoattractant protein-1 (MCP-1), vascular endothelial growth factor (VEGF), and epidermal growth factor (EGF). Insulin resistance was calculated with the homeostatic model assessment method of insulin resistance (HOMA-IR) [28]. The same patients were visited and subjected to the same blood determinations after 1 and 4 months from dietary treatment. We established a cut-off of 5% at 4 months to indicate the efficacy of treatment.

2.2. Blood Sample Collection and Routine Work-Up. Blood was collected, after 12–14 h fasting, by antecubital venous puncture. Serum or plasma samples were obtained by centrifugation. Routine analyses, including blood count, PT, PTT and fibrinogen, fasting glucose, total cholesterol, HDL and LDL, triglycerides, AST, ALT, γGT, and LDH, were obtained from fresh samples, whereas aliquots of serum or citrated plasma were frozen at −80° for subsequent laboratory determinations (insulin, adipokines, cytokines, hsCRP, cystatin C, and PAI-1).

Blood count analysis was performed using ADVIA 2120 (Siemens, USA); PT, aPTT, and fibrinogen were performed on BCS XP (Siemens, USA); blood glucose, total cholesterol, HDL and LDL, triglycerides, AST, ALT, γGT, and LDH were performed on Cobas 6000 (Roche, Switzerland). All the above-mentioned assays were carried out according to the manufacturer's instructions.

Reference values of each measured analyte are reported in the supplementary table (see Supplementary Material available online at http://dx.doi.org/10.1155/2014/750860).

2.3. Measurements of Serum Cystatin C and hsCRP. The determinations of serum concentrations of cystatin C and hsCRP were carried out using a nephelometer BNII (Siemens, USA) by immunonephelometric methods, according to

the manufacturer's instructions (Penia N Latex Cystatin C kit and CardioPhase hsCRP kit, Siemens, USA, resp.).

2.4. Measurement of Serum Cytokines. The serum concentrations of 12 different cytokines and growth factors (IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IFNγ, TNFα, MCP-1, VEGF, and EGF) were simultaneously determined using the biochip analyser Evidence Investigator (Randox Labs, UK) and the "Cytokine Array I and High sensitivity" kit, Randox, UK, according to the manufacturer's instructions. Briefly, the principle of this multianalyte testing relies on a sandwich ELISA, in which the analytes of interest are captured by specific antibodies bound to discrete regions of the surface chemistry of the biochip; horseradish peroxidase (HRP) labeled secondary antibodies, which specifically recognize the analytes, trigger a luminol-based electrochemiluminescent signal emission, registered by a CCD camera and quantified by a software.

2.5. Measurement of Serum Insulin and Adipokines. The serum concentration of insulin was measured by the ADVIA Centaur Immunoassay system (Siemens, USA), using a chemiluminescent immunoassay. Serum adipokines were determined by sandwich ELISA assays on the automated ELISA analyser Triturus (Grifols, USA), with the kits produced by Mediagnost, Germany (Adiponectin E09, sensitive leptin E077, resistin E50). Visfatin was determined by Visfatin C-Terminal Human, EIA Kit (Phoenix).

2.6. Statistical Analysis. Each quantitative trait was tested for normality using the Shapiro-Wilk normality test and, when required, it has been log-transformed. Any continuous variable was indicated as median and interquartile range. The nonparametric Friedman and Wilcoxon tests were used to evaluate the differences between the intragroup and anthropometric variables and laboratory parameters during the time of the study. A significance level of $P < 0.05$ was set for a type I error in all analyses. Multivariate analysis was performed by incorporating age and sex as covariates using the linear regression procedure. All statistical procedures were performed by using the SPSS 20.0 software for Mac.

3. Results

The characteristics of the population studied are summarized in Table 1. Most participants (74%) were women, and the mean age was 52 years. At baseline, the population enrolled was affected by grade I, grade II, and severe obesity (34.8%, 43.5%, 21.7%, resp.), and the mean BMI was 37 kg/m^2. Data on body composition indicate the contribution of adiposity to body weight. Comparison of all the parameters examined at time of 0, 1, and 4 months from diet treatment is shown in Table 2. During the follow-up, body weight and BMI significantly decreased ($P < 0.001$) after calorie restriction, with an average reduction of body weight of 3.4% at 1 month and 5.8% at 4 months after dieting. After the 4-month dietary intervention, in fact, body weight loss redistributed the status of patients and their proportions, as overweight (17.4%) and grade I (34.8%) and grade II (26.1%) obese, while the percentage of patients with severe obesity had no variation (21.7%).

Table 1: Demographic and anthropometric features of obese patients at the enrollment.

	Patients
Race	Caucasian
N	23
Female	17
Age (yrs)	52 (46–61)
Height (cm)	155 (153–167)
BMI (Kg/m^2)	37 (32–39)
FM (%)	43.5 (34.5–46.0)
LM (%)	37.0 (33.0–43.7)
FM/LM	1.2 (0.8–1.4)

FM: fat mass; LM: lean mass, as calculated by bioelectrical impedance analysis. Continuous variables are expressed as median and quartiles.

Weight loss led to an improvement in insulin sensitivity, as indicated by a decrement of both insulin ($P = 0.037$) and HOMA-IR index ($P = 0.026$). LDH showed a decrement after 4 months of treatment ($P = 0.023$), whereas leptin was the biomarker that varied more significantly throughout the whole time period ($P = 0.008$). Also, to note, EGF showed a significant increase ($P = 0.013$). Some other analytes showed an interesting trend of variability. In particular, a decremental trend has been observed for IL-6 and VEGF and for prothrombotic risk parameters, such as fibrinogen and PAI-1 (Table 2). To strengthen the precocious variation of the parameters examined, we compared all the variables after only one month of dietary intervention. Statistical analysis showed significant variations of insulin, HOMA-IR, leptin, resistin, EGF, and total cholesterol, confirming leptin as the most sensitive marker (Table 3).

Then, we analyzed the effect of weight loss on biochemical parameters after 4 months, compared subjects who obtained a decrease in their body weight greater than 5% with those who did not, and arbitrarily used a weight loss >5% as a group variable. Notably, we observed a more significant variation of insulin ($P = 0.025$), leptin ($P = 0.023$), and EGF ($P = 0.035$) in patients achieving a greater body weight loss (Table 4).

4. Discussion

Diet represents the most widespread tool to reduce weight in the obese individuals [29]. It has been shown that even a moderate weight loss of about 10% or less contributed to several health advantages that include improvement in metabolic parameters, reduction of blood pressure, and increase in longevity [25, 26]. Also, there are circumstantial lines of evidence that calorie restriction improves insulin sensitivity and reduces systemic inflammation [30–33].

Several reports [27, 34–36] have emphasized the benefits of weight loss in obese individuals by evaluating physical and biochemical markers after at least 6 months from diet introduction, whereas little is known about possible earlier

Table 2: Comparison of demographic, anthropometric, clinical, and biochemical features of obese patients at baseline and during follow-up.

	Baseline	1 month	4 months	*P*
Weight (Kg)	91 (82–112)	87 (83–101)	87 (78–98)	<0.001
BMI (Kg/m^2)	37 (32–39)	36 (31–38)	35 (31–38)	<0.001
Total cholesterol (mg/dL)	199 (172–225)	190 (151–222)	209 (168–218)	0.249
HDL (mg/dL)	50 (38–57)	44 (39–53)	49 (41–57)	0.073
LDL (mg/dL)	131 (95.0–160.0)	117 (95.0–153.0)	120 (95.0–149)	0.444
Triglycerides (mg/dL)	105 (66–169)	111 (76–132)	95 (83–149)	0.420
Fasting glucose (mg/dL)	97 (91–107)	96 (91–99)	94 (87–104)	0.168
Basal insulin (μU/mL)	18.0 (13.0–27.0)	15.0 (10.0–20.0)	14.0 (10.0–28.0)	0.037
HOMA-IR	4.0 (3.0–6.0)	4.0 (2.0–5.0)	3.0 (3.0–6.0)	0.026
γGT (U/L)	22.0 (15.0–44.0)	21.0 (14.0–40.0)	19.0 (13.0–65.0)	0.299
AST (U/L)	23.0 (17.0–27.0)	21.0 (18.0–24.0)	19.0 (16.0–24.0)	0.238
ALT (U/L)	27.0 (19.0–34.0)	25.0 (18.0–35.0)	22.0 (17.0–33.0)	0.220
LDH (U/L)	345 (326–397)	350.0 (329–403)	323 (296–376)	0.023
Adiponectin (μg/mL)	5.0 (3.0–7.0)	4.0 (3.0–6.0)	5.0 (3.0–7.0)	0.923
Leptin (ng/mL)	47.0 (31.0–70.0)	35.0 (22.0–46.0)	36.0 (27.0–44.0)	0.008
Resistin (ng/mL)	5.0 (4.0–6.0)	5.0 (5.0–7.0)	5.0 (4.0–7.0)	0.267
Visfatin (ng/mL)	7.0 (5.0–8.0)	7.0 (6.0–9.0)	7.0 (6.0–9.0)	0.559
Cystatin C (mg/dL)	0.79 (0.71–0.85)	0.78 (0.67–0.85)	0.78 (0.72–0.85)	0.640
PT (%)	102 (98–104)	103 (96–104)	104 (99–110)	0.211
aPTT (sec)	27 (26–28)	28 (27–30)	27 (25–29)	0.001
Fibrinogen (mg/dL)	325 (274–369)	324 (287–349)	308 (260–341)	0.676
PAI-1 (U/mL)	5.0 (2.0–6.0)	3.0 (1.0–6.0)	3.0 (1.0–4.0)	0.610
hsCRP (mg/dL)	2.8 (1.8–7.0)	2.8 (1.61–5.2)	4.0 (1.2–6.4)	0.568
IL-2 (pg/mL)	0.0 (0.0–5.0)	3.0 (0.0–5.0)	3.0 (0.0–6.0)	0.154
IL-4 (pg/mL)	0.0 (0.0–2.0)	0.0 (0.0–2.0)	0.0 (0.0–0.0)	0.409
IL-6 (pg/mL)	1.3 (1.0–2.0)	1.3 (1.0–2.0)	1.0 (1.0–2.0)	0.394
IL-8 (pg/mL)	10.0 (7.0–28.0)	14.0 (6.0–38.0)	14.0 (10.0–25.0)	0.535
IL-10 (pg/mL)	0.0	0.0	0.0	—
VEGF (pg/mL)	193 (98–361)	178 (101–283)	174 (90–267)	0.568
IFNγ (pg/mL)	2.0 (0.0–4.0)	2.0 (0.0–4.0)	3.0 (0.0–6.0)	0.486
TNFα (pg/mL)	3.0 (3.0–5.0)	3.0 (3.0–6.0)	4.0 (3.0–7.0)	0.867
IL-1α (pg/mL)	0.0	0.0	0.0	—
IL-1β (pg/mL)	0.8 ± 2.0*	0.7 ± 2.3*	0.8 ± 2.7*	0.662
MCP-1 (pg/mL)	351 (206–395)	352 (232–515)	367 (214–485)	0.302
EGF (pg/mL)	86 (68–130)	114 (84–144)	97 (68–139)	0.013

Nonparametric Friedman test has been used for comparisons at three times. All variables are expressed as median and quartiles. *These data are expressed as average ± SD.

Table 3: Significant variations of anthropometric, clinical, and biochemical features of obese patients after one month of calorie restriction.

	Baseline	1 month	*P*
Weight (Kg)	91 (82–112)	87 (83–101)	<0.001
BMI (Kg/m^2)	37 (32–39)	36 (31–38)	<0.001
Basal insulin (μU/mL)	18.0 (13.0–27.0)	15.0 (10.0–20.0)	0.011
HOMA-IR	4.0 (3.0–6.0)	4.0 (2.0–5.0)	0.006
Leptin (ng/mL)	46.0 (31.0–70.0)	35.0 (22.0–46.0)	<0.001
Resistin (ng/mL)	5.0 (4.0–6.0)	5.0 (5.0–7.0)	0.017
Total cholesterol (mg/dL)	199 (172–225)	190 (148–220)	0.047
EGF (pg/mL)	86 (68–130)	114 (84–144)	0.018

Nonparametric Wilcoxon test has been used for comparisons at two times. All variables are expressed as median and quartiles.

Table 4: Effect of weight loss on biochemical parameters.

	Weight loss >5%	Weight loss ≤5%	Beta	t	P
Total cholesterol (mg/dL)	206 (184–226)	177 (146–218)	0.338	1.699	0.106
HDL (mg/dL)	52 (44–68)	49 (45–54)	0.075	0.321	0.752
Triglycerides (mg/dL)	89 (79–154)	101 (71–134)	0.020	0.081	0.936
Fasting glucose (mg/dL)	95 (88–119)	94 (86–103)	0.300	1.181	0.252
Basal insulin (μU/mL)	10.0 (8.7–15.7)	24.0 (12.0–28.5)	−0.506	−2.430	0.025
HOMA-IR	3.0 (2.0–3.2)	5.0 (3.0–6.0)	−0.435	−2.040	0.056
γGT (U/L)	19.5 (14.2–56.0)	17.0 (11.0–81.0)	0.123	0.487	0.632
AST (U/L)	20.5 (15.7–24.2)	18.0 (16.0–24.0)	0.144	0.646	0.526
ALT (U/L)	19.0 (17.2–32.0)	23.0 (14.5–33.5)	0.329	1.317	0.203
LDH (U/L)	323 (298–389)	334 (295–363)	−0.141	−0.587	0.564
Adiponectin (μg/mL)	6.0 (4.0–7.25)	4.0 (3.0–5.0)	0.298	1.164	0.259
Leptin (ng/mL)	31.5 (19–40.0)	41.0 (31.5–53.5)	−0.575	−2.478	0.023
Resistin (ng/mL)	5.0 (4.0–8.0)	6.0 (4.0–9.0)	−0.258	−0.940	0.359
Visfatin (ng/mL)	7.5 (6.0–10.0)	7.0 (6.0–8.5)	0.088	0.353	0.729
Cystatin C (mg/dL)	1.0 (1.0-1.0)	1.0 (1.0-1.0)	—	—	—
PT (%)	105 (102–111)	103 (97–108)	0.079	0.321	0.752
aPTT (sec)	27 (25–28)	27 (25–29)	−0.121	−0.554	0.586
Fibrinogen (mg/dL)	297 (261–346)	319 (244–339)	−0.068	−0.271	0.790
PAI-1 (U/mL)	2.5 (1.0–4.5)	3.0 (1.5–4.0)	0.010	0.038	0.970
hsCRP (mg/dL)	4.5 (1.0–9.2)	4.0 (2.0–5.5)	0.014	0.065	0.949
IL-2 (pg/mL)	3.5 (0.0–5.2)	3.0 (0.0–6.0)	0.001	0.002	0.999
IL-4 (pg/mL)	0.0 (0.0–2.0)	0.0 (0.0–2.0)	—	—	—
IL-6 (pg/mL)	1.0 (1.0–2.0)	1.0 (1.0–2.0)	0.058	0.256	0.801
IL-8 (pg/mL)	15.0 (12.5–28.2)	12.0 (9.0–23.0)	0.070	0.298	0.769
IL-10 (pg/mL)	0.0	0.0	—	—	—
VEGF (pg/mL)	230 (69–347)	157 (100–238)	−0.025	−0.105	0.917
IFNγ (pg/mL)	4.0 (0.7–9.7)	2.0 (0.0–4.0)	0.318	1.159	0.271
TNFα (pg/mL)	4.0 (2.7–7.2)	5.0 (4.0–7.5)	−0.191	−0.887	0.386
IL-1α (pg/mL)	0.0	0.0	—	—	—
IL-1β (pg/mL)	1.4 ± 4.2*	0.3 ± 0.7*	—	—	—
MCP-1 (pg/mL)	372 (228–547)	338 (203–444)	0.243	1.111	0.280
EGF (pg/mL)	77 (54.5–106.2)	121 (87–156)	−0.468	−2.273	0.035

Multivariate linear regression analysis has been adjusted for gender and age. All variables are expressed as median and quartiles.* These data are expressed as average ± SD.

changes of these indexes. In particular, by comparing different hypocaloric diets, the Mediterranean diet has proved to be associated with a greater improvement of insulin sensitivity parameters [27]. The innovative aspects of our study consisted in the short-term assessment, in obese subjects, of clinical and laboratory parameters after a hypocaloric, Mediterranean diet and the achievement of a generally modest weight loss.

In our study, the efficacy of dietary treatment was proved by an average reduction of body weight of 3.4% at 1 month and 5.8% at 4 months and by a statistically significant reduction in BMI. Most studies conducted so far have considered a minimum of 5% body weight loss indispensable to obtain beneficial effects [25, 26, 37]. Interestingly, our findings show that, even after a smaller decrement of body weight, diet is associated with a precocious, statistically significant reduction of insulin levels and HOMA-IR index that are compatible with an early improvement of insulin sensitivity. We cannot however exclude or quantify the possible contribution of physical activity to this issue.

We also examined cytokines and adipokines for their link to the pathophysiology of obesity and insulin resistance. It is well known that the adipose tissue is an endocrine organ with a wide, biologically active secretome [38, 39] and that the adipose tissue of obese individuals is characterized by an increased expression and/or secretion of several proinflammatory cytokines, as well as of adipokines known to promote inflammation, atherogenesis, and insulin

resistance, whereas the biosynthesis of anti-inflammatory, antiatherogenic, and insulin-sensitising adipokines, such as adiponectin, is decreased [40–42].

Several studies have shown that increased circulating leptin levels and increased adiposity are associated with leptin resistance and that this condition can contribute to the onset and/or maintenance of obesity [43–46]. In our study, we observed, throughout the follow-up, a significant decrease in circulating levels of leptin, which showed a statistically significant positive correlation with both BMI and insulin. Although insulin and leptin resistance have been widely described in the obese subjects, the relationships between these two hormones are complex and not fully understood, and evidence exists that they are associated with body fat through different mechanisms [47, 48]. No statistical variations of other adipokines or cytokines have been observed after dietary treatment in the whole time period of the study. Other studies with comparable or even greater sample size had obtained similar results and emphasized a statistically significant decrement in leptin levels following diet [49–51]. Leptin, but not adiponectin, resistin, RBP-4, or cytokines, has been correlated with calorie restriction and weight loss in a recent study [52], while an improvement in adipokine profile was observed in severely obese women, only after a 5 to 10% weight loss [37].

Based on previous findings and on the consideration that BMI is a good predictor of cardiovascular disease risk [53, 54], we hypothesized that weight loss could be associated with an improvement in hemostatic parameters. Due to the small sample size, our results showed nonstatistically significant differences, as also reported by other works that investigated an even greater number of patients [55, 56].

An intriguing and apparently equivocal finding in our study is the precocious increase of circulating EGF levels after diet introduction, while EGF decreases in the patient group that had lost >5% of body weight, in association with an improvement in insulin sensitivity. Salivary glands are the major source of circulating EGF, a growth factor implicated in the control of cell proliferation and differentiation [57]. During fasting, salivary and plasma EGF physiologically increase [58] to inhibit gastric acid secretion and to preserve the oroesophageal and gastrointestinal mucosal integrity. We hypothesize that the increase of EGF observed in our study is coherent with the food restriction state and with the intestinal adaptation to diet. The increase in EGF may exert insulin-like biological activities in tissues expressing high levels of EGF receptors (EGFR), such as fat and skeletal muscle [59]. By binding to EGFR, a receptor belonging to the tyrosine kinase receptor family, like the insulin receptor [60], EGF can amplify the downstream signaling of insulin through the recruitment of additional PI3-kinase pools in tissues, in which both cognate receptors and the downstream signaling molecules are all abundantly expressed, leading to an EGF-induced translocation of GLUT4 on the plasma membranes and the stimulation of glucose uptake in target tissues [59]. These molecular mechanisms are particularly important in insulin-resistant states, including obesity, in which the increase of EGF due to food restriction may trigger insulin-like compensatory mechanisms. It is also known that insulin regulates EGF expression [61]. This explains the parallel decrease of both insulin and EGF in patients who achieved a more consistent weight loss, in which insulin sensitivity has been improved or reestablished.

LDH is an intracellular enzyme that is released into the bloodstream when tissues are destroyed or injured. Therefore, LDH is considered an important clinical marker of tissue or cell integrity. In the obese, the adipose tissue is dysfunctional [62, 63] and exhibits a reduced capacity to store and retain nonesterified fatty acids (NEFA), leading to an increase in NEFA circulating levels, thereby promoting the development of lipotoxicity in peripheral tissues [64]. In this study, we observed a significant reduction of circulating levels of LDH following diet, a condition compatible with an attenuation of the dysfunctional, proinflammatory mechanisms that characterizes obesity.

5. Conclusions

On the whole, our study confirms the efficacy of a moderate hypocaloric Mediterranean diet to improve insulin sensitivity in obese patients. The novelty of our findings is that calorie restriction determines a precocious variation of both insulin and leptin even after achieving a modest weight loss. In addition to these traditional laboratory parameters, LDH and EGF have emerged as novel obesity-related markers, pathophysiologically linked to obesity and insulin resistance, that deserve further attention. In particular, the precocious increment of EGF might be clinically useful as an early predictor for a positive evolution of the metabolic response following calorie restriction.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Total Adiponectin Is Inversely Associated with Platelet Activation and CHA_2DS_2-VASc Score in Anticoagulated Patients with Atrial Fibrillation

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Background. Adiponectin (APN) possesses anti-inflammatory and antiatherogenic effects. Atrial fibrillation (AF) is burdened by enhanced systemic inflammation and platelet activation, as documented by increased blood levels of soluble CD40L (sCD40L). The interplay between APN and platelet activation in AF is still undefined. *Materials and Methods*. Circulating levels of APN and sCD40L were measured in 257 anticoagulated nonvalvular AF patients. Exclusion criteria were as follows: prosthetic heart valves, cardiac revascularization in the previous year, severe cognitive impairment, chronic infectious or autoimmune diseases, and active cancer. *Results*. Mean age was 72.9 (±8.7) years and 41.6% were female. Serum APN and plasmatic sCD40L were inversely correlated (R −0.626, $P < 0.001$). A progressive increase of sCD40L across tertiles of CHA_2DS_2-VASc score was observed (rS 0.473, $P < 0.001$), whilst APN was inversely correlated (rS −0.463, $P < 0.001$). A multivariable linear regression analysis showed that CHA_2DS_2-VASc score (B −0.227, $P < 0.001$) and sCD40L (B −0.524, $P < 0.001$) correlated to APN. *Conclusions*. AF patients at high risk of stroke disclose low and high levels of APN and sCD40L, respectively, suggesting a role for APN if it favors platelet activation in vivo in this clinical setting. Enhancing APN levels may be a future goal to reduce the risk of vascular outcomes in AF patients.

1. Introduction

Atrial fibrillation (AF) is the most frequent supraventricular cardiac arrhythmia in the general population. Patients affected by AF, despite the recent introduction of novel oral anticoagulants, show an increased risk for ischemic vascular complications, such as ischemic stroke and cardiovascular mortality [1].

Several evidences suggest that AF is burdened by an enhanced systemic inflammatory status [2] and platelet activation [3, 4], as shown by the enhanced release of soluble CD40 ligand (sCD40L), which may affect AF-related thromboembolic events [5].

AF is characterized by the simultaneous presence of different atherosclerotic risk factors, frequently represented by arterial hypertension, diabetes, obesity, and dyslipidemia [6]. In particular, obesity is a well-known recognized risk factor for developing AF, and more recently weight loss has been associated with improved cardiac symptoms and reduced cardiac remodeling in AF patients [7, 8].

Adiponectin (APN) is the most abundant adipokine produced by adipose tissue, acting as an insulin-sensitizer

and anti-inflammatory molecule [9, 10]. In addition to its metabolic properties, APN exerts antiatherogenic effects [11], and reduced serum APN levels have been found in patients with obesity, insulin resistance, and type 2 diabetes [12].

Data regarding APN levels in AF are controversial. Low serum APN levels have been found in paroxysmal AF patients compared to controls [13], whilst higher APN levels have been described in patients with permanent AF, compared to paroxysmal AF and controls [14]. Recently, Hernandez-Romero et al. have shown an association between low APN levels and cardiovascular outcomes in patients affected by AF [15]. Among the mechanisms accounting for such association, the interplay between APN and platelet activation could be considered as platelets play a key role in precipitating acute coronary and cerebrovascular disease [16]. Thus, APN is an antioxidant molecule, which inhibits platelet activation via lowering platelet oxidative stress [17, 18]. So far, data on APN interplay with platelet activation in AF patients are lacking. The aim of the study has been to examine the relationship between APN and platelet activation, as assessed by sCD40L, and to evaluate the association with CHA_2DS_2-VASc score in a cohort of anticoagulated nonvalvular AF patients.

2. Material and Methods

2.1. Study Design and Patient Selection. The study included 257 consecutive patients with AF who were referred to the Atherothrombosis Center of the Department of Internal Medicine and Medical Specialties of "Sapienza" University of Rome. All patients were treated with oral vitamin K antagonists according to CHA_2DS_2-VASc score [19] and the international normalized ratio was maintained in a therapeutic range of 2.0-3.0.

Exclusion criteria included presence of prosthetic heart valves, cardiac revascularization in the previous year, severe cognitive impairment (Alzheimer's disease and Parkinson's disease), chronic infectious diseases, autoimmune systemic diseases, and active cancer.

At enrollment, medical history, anthropometric data, and electrocardiogram were recorded and a sample of blood was collected from all patients. Arterial hypertension was defined as elevated blood pressure (≥140/≥90 mmHg) or taking antihypertensive therapy [20] regimen; diabetes was defined as a casual plasma glucose ≥200 mg/dL (11.1 mmol/L), fasting plasma glucose ≥126 mg/dL (7.0 mmol/L), or antidiabetic treatment [21]. Heart failure (HF) was defined as the presence of signs and symptoms typical of heart failure or reduced ejection fraction (EF ≤ 40%) [22]. Metabolic syndrome (MetS) was defined according to modified ATP-III criteria: abdominal obesity, given as waist circumference (in men >102 cm/>40, in women >88 cm/>35); triglycerides ≥150 mg/dL, HDL cholesterol (men <40 mg/dL, women <50 mg/dL); blood pressure ≥130/≥85 mmHg; fasting glucose ≥100 mg/dL [23].

All patients provided a written informed consent. The study protocol was approved by the local ethical board of "Sapienza" University of Rome and was carried out according to the principles of the Declaration of Helsinki [24].

2.2. Laboratory Analysis. Total serum APN levels were measured with a commercial immunoassay (Tema Ricerca, Italy) and expressed as ng/mL. Intra-assay and interassay coefficients of variation were 6% and 8%, respectively.

Platelet activation was assessed by the release of sCD40L. Blood samples were collected without stasis to minimize platelet activation from subjects who had fasted for at least 12 hours, directly mixed in a vacutainer (Vacutainer Systems, Belliver Industrial Estate) with 1 part of 3,8% Na citrate (ratio 9 : 1) and immediately centrifuged for 20 minutes at 2000 rpm at −4°C. Plasma samples were stored at −80°C until use.

Plasma levels of sCD40L were evaluated by immunoassay (Quantikine CD40 ligand, R&D Systems) and expressed as ng/mL. Intra-assay and interassay coefficients of variation were 6% and 7%, respectively.

2.3. Statistical Analysis. Categorical variables were reported as counts (percentages) and continuous variables as mean ± standard deviation (SD) or median and interquartile range (IQR) unless otherwise indicated. Independence of categorical variables was tested by χ^2 test. Normal distribution of parameters was assessed by Kolmogorov-Smirnov test. Student's unpaired t-test and Pearson product-moment correlation analysis were used for normally distributed continuous variables. After dividing population according to tertiles of CHA_2DS_2-VASc score, APN and sCD40L were analyzed. Group comparisons were performed using analysis of variance (ANOVA). Appropriate nonparametric tests (Mann-Whitney U-test, Kruskal-Wallis test, and Spearman rank correlation test [rS]) were employed for all the other variables. Only P values lower than 0.05 were considered as statistically significant. All tests were two-tailed and analyses were performed using computer software packages (SPSS-18.0, SPSS Inc.).

2.4. Sample Size. We calculated that 48 patients per group were required to have a 90% chance of detecting, as significant at the 5% level, a difference for APN levels between groups of 2 ng/mL with SD = 3 ng/mL.

3. Results

Baseline characteristics of all patients are reported in Table 1. Mean age was 72.9 (±8.7) years and 41.6% were female. One-hundred sixteen patients (45.1%) had paroxysmal AF, whilst 141 (54.9%) had persistent/permanent AF.

Median adiponectin value was 5.6 [3.2–8.6] ng/mL and median sCD40L was 49 [40–70] ng/mL. A significant inverse correlation between adiponectin and sCD40L was found (R −0.626, $P < 0.001$) (Figure 1).

After dividing population according to tertiles of CHA_2DS_2-VASc score, a significant difference across tertiles was found both for sCD40L ($P < 0.001$) and APN ($P < 0.001$) (Table 2) (Figures 2(a) and 2(b)).

In particular, a progressive increase of sCD40L across tertiles of CHA_2DS_2-VASc score was observed (rS 0.473, $P < 0.001$) (Table 2). On the contrary, APN inversely correlated

Table 1: Baseline characteristics.

Anthropometric and metabolic data	
Age (years)	72.9 ± 8.7
Female gender (%)	41.6
Body mass index (kg/m^2)	27.0 ± 4.3
CHA_2DS_2-VASc score[#]	3 (2–4)
Adiponectin[#] (ng/mL)	5.6 (3.2–8.6)
sCD40L[#] (ng/mL)	49 (40–70)
Cardiovascular risk factors	
Hypertension (%)	88.3
Diabetes mellitus (%)	17.9
Heart failure (%)	16.7
History of stroke/TIA (%)	14.0
History of MI (%)	23.0
Metabolic syndrome (%)	51.0
Concomitant therapies	
(i) Antiplatelets (%)	11.3
(ii) ACE inhibitor/ARBs (%)	68.1
(iii) β blockers (%)	45.1
(iv) Calcium channel blockers (%)	33.9
(v) Statins (%)	45.5
(vi) Antiarrhythmic drugs (%)	34.2

[#]Data are expressed as median and interquartile range.
sCD40L: soluble CD40 ligand, TIA: transient ischemic attack, MI: myocardial infarction, ACE: angiotensin converting enzyme, and ARBs: angiotensin receptor blockers.

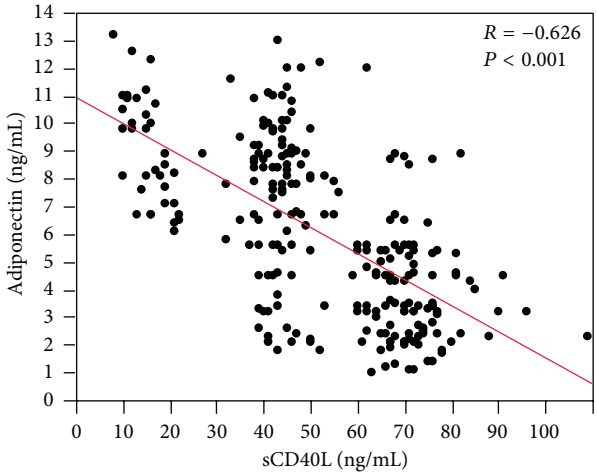

Figure 1: Linear regression analysis between sCD40L and adiponectin levels.

with tertiles of CHA_2DS_2-VASc score (rS −0.463, $P < 0.001$) (Table 2).

After adjustment for potential confounding factors, such as age, BMI, smoking habits, and diabetes, multivariable linear regression analysis showed that only CHA_2DS_2-VASc score (R^2 0.431, B −0.227, $P < 0.001$) and sCD40L (R^2 0.392, B −0.524, $P < 0.001$) were independently correlated to adiponectin.

Table 2: Median values of adiponectin and sCD40L according to tertiles of CHA_2DS_2-VASc score.

	CHA_2DS_2-VASc score			P value
	1st tertile	2nd tertile	3rd tertile	
Adiponectin	8.4 (5.5–9.2)	6.5 (4.5–8.7)	3.1 (2.3–4.3)	<**0.001**
sCD40L	44 (40.0–47.5)	45 (22–68)	70 (67–74)	<**0.001**

sCD40L: soluble CD40 ligand.

4. Discussion

The present study shows an inverse relationship between serum APN and CHA_2DS_2-VASc score, suggesting that lower antioxidant and higher inflammatory conditions are detectable in patients at a higher risk of stroke; the inverse relation between APN and sCD40L also suggests a role for oxidative stress in enhancing platelet activation in vivo.

Experimental and observational studies demonstrated that APN is directly involved in generating cardiovascular ischemic complications in the general population [25] and in different clinical settings at risk of vascular complications [15, 26–28]. Evidence derived from the in vitro study suggested that APN was able to inhibit the macrophage tissue factor, a key molecule promoting thrombus formation in disrupted plaques [29]. In APN-knockout mice, APN deficiency was associated with enhanced thrombus formation and platelet aggregation [11]. The interplay between APN and platelet activation has been explored in patients at risk of vascular complications [30, 31]. Thus, patients with MetS express receptors for APN on the platelet surface; platelet incubation with APN resulted in platelet aggregation inhibition and impaired CD40L release [31]. Similar results were observed in patients with type 2 diabetes [32], in which spontaneous platelet aggregation was inhibited by APN.

Clinical studies demonstrated that APN levels correlated with coronary artery disease severity [27], atherothrombotic and lacunar stroke types in men [28], and with incident heart failure in the physicians' health study [26]. Concerning AF, a recent study showed that APN levels were associated with cardiovascular events in anticoagulated AF female patients [15]. To investigate the mechanism potentially accounting for such inverse association, we analyzed the interplay between APN and sCD40L, which is a marker of platelet activation as it derives prevalently from CD40L released by the activated platelet [33, 34]. The inverse correlation between APN and sCD40L may provide novel insights on in vivo platelet activation in this setting as it suggests that the antioxidant status predisposes platelet activation. Such hypothesis is biologically plausible as previous studies consistently showed that oxidative stress is implicated in platelet activation via several mechanisms including formation of isoprostanes, which are chemically stable eicosanoids with proaggregating property [35], and an inactivation of nitric oxide, a powerful antioxidant molecule [36]. Also, platelet incubation with antioxidants different than APN similarly results in inhibition of platelet aggregation [37]. A previous study from our group [5] demonstrated that platelet activation, as assessed by circulating levels of sCD40L, is predictive of vascular outcomes

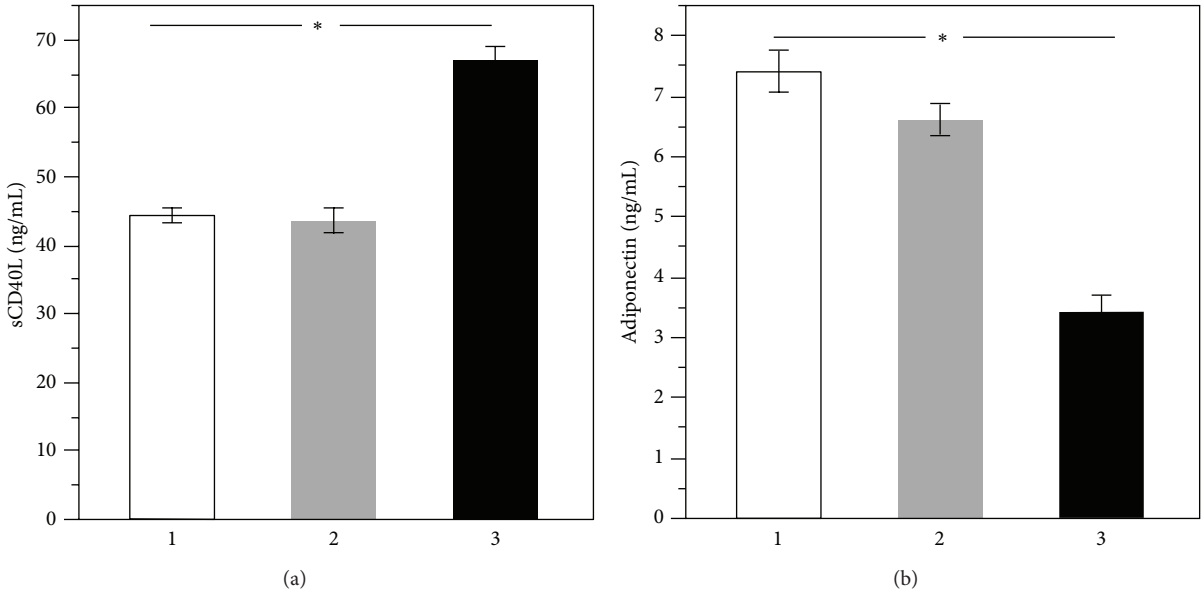

Figure 2: Median values of sCD40L (a) and adiponectin (b) according to tertiles of CHA_2DS_2-VASc score.

in AF patients but the mechanism accounting for platelet activation was not explored. The present study provides insight into these findings suggesting that the antioxidant status may account for platelet activation in AF.

Another finding of the present study is the inverse correlation between APN and CHA_2DS_2-VASc score indicating that the risk of stroke is higher and the antioxidant status is lower. This finding is consistent with a previous study showing that serum levels of vitamin E, another molecule with antioxidant property, are inversely related to CHA_2DS_2-VASc score and predict cardiovascular outcomes in AF patients [38].

From our data it is, therefore, arguable that patients at a higher risk of stroke disclose a lower antioxidant status in association with platelet activation, both changes being potentially implicated in precipitating vascular outcomes by favoring atherosclerotic progression and thrombosis.

In conclusion, the present study shows that AF patients at high risk of stroke disclose low and high levels of APN and sCD40L, respectively, suggesting a role for APN in favoring platelet activation in vivo in this clinical setting. Enhancing APN levels may be a future goal to reduce the risk of vascular outcomes in AF patients.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Camilla Calvieri and Giacomo Frati have equal contribution.

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Research Article

Role of TGF-β Pathway Polymorphisms in Sporadic Thoracic Aortic Aneurysm: rs900 TGF-β2 Is a Marker of Differential Gender Susceptibility

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Thoracic aortic aneurysm (TAA) is a progressive disorder involving gradual dilation of ascending and/or descending thoracic aorta with dissection or rupture as complications. It occurs as sporadic or defined syndromes/familial forms.Genetic, molecular and cellular mechanims of sporadic TAA forms are poorly characterized and known. Thus, our interest has been focused on investigating the role of genetic variants of transforming growth factor-β (TGF-β) pathways in TAA risk. On the other hand, no data on the role of genetic variants of TGF-β pathway in sporadic TAA exist until now. In addition, other cytokines, including IL-10, orchestrate TAA pathophysiology. Their balance determines the ultimate fate of the aortic wall as healing atherosclerosis or aneurysm formation. Thus, in this paper it was analyzed the role of ten polymorphisms of genes encoding TGF-β isoforms and receptors, and IL-10 in sporadic TAA. Our study included cases affected by sporadic TAA and two control groups. The most relevant finding obtained allows us to propose that rs900 TGF-β2 SNP is associated with sporadic TAA in women. This might open new perspectives for the analysis of sporadic TAA susceptibility factors and prevention.

1. Introduction

Thoracic aortic aneurysm (TAA) is a pathological widening of aorta resulting from degeneration of the extracellular matrix and loss of smooth muscle cells in the tunica media. TAA has different etiological causes, including monogenic syndromes (such as Marfan and Loeys-Dietz syndromes), bicuspid aortic valve (BAV) disease, and idiopathic causes [1, 2].

The pathogenesis of TAA in monogenic syndromes has been extensively studied [3]. The attested evidence, indeed, suggests the deregulation of transforming growth factor-β (TGF-β) signaling characterized by its enhanced function and damaged TGF-β receptors as their common and typical feature [4, 5].

The TGF-β family is constituted by TGF-β1, TGF-β2, and TGF-β3 members, which are pleiotropic secreted cytokines having a broad spectrum of biologic functions. Among these, the TGF-β1 has numerous cellular functions, including cell growth, cell proliferation, cell differentiation, and apoptosis. In humans, TGF-β gene product's effects can stimulate or inhibite cell growth depending cellular and tissue targets. TGF-β1 can modulate cell differentiation and proliferation in auto- or paracrine manner [6]. In vascular smooth muscle cells, TGF-β can upregulate fibronectin and connective tissue growth factor expression via activation of small mothers against decapentaplegic (Smad) proteins [7]. As a consequence, it can promote the deposition of components of extracellular matrix (ECM) [8]. Furthermore, its action depends on the interaction with specific receptors, such as TGF-β receptor (TGF-βR) I and TGF-βRII, glycoproteins of 55 kDa and 70 kDa, respectively, with core polypeptides of 500–570 amino acids [9].

TGF-β actually is considered as a crucial player in vascular remodelling, able to alter both structure and ECM composition. In Marfan syndrome, the fibrillin-1 gene mutations seem to influence the bioavailability of active TGF-β. In addition, mutations in the TGF-β receptors also impair the signalling cascade in other Marfan syndrome related disorders, including Loeys-Dietz syndrome, familial TAAs, and aortic dissection. Furthermore, mutations in Notch gene homolog 1 (NOTCH1) and Notch1 pathway, mainly identified in TAA patients with BAV, seem to influence TGF-β crosstalk [10].

In contrast, molecular and genetics mechanisms of the nonfamilial TAA forms, representing the major number of cases of TAAs, remain largely unknown [11]. Different roles of TGF-β pathways in tissue remodelling mechanisms have been reported in both sporadic thoracic and abdominal aneurysms [8]. In particular, both loss and gain of functional TGF-β signalling have been described as predisposing factors for both sporadic TAA development and dissection. The paradoxical effect of TGF-β leading to enhanced connective matrix degradation through metalloproteinase activation has been principally observed in the nonsyndromic cases of familial TAAs and dissection [4]. In addition, TGFBR1 and TGFBR2 losses induced by functional mutations have been associated with both familial syndromic and nonsyndromic TAAs [12–14]. This altered condition of TGF-β signalling has been demonstrated to induce unusually the activation of TGF-β mediated connective matrix degradation [4].

Furthermore, vascular remodelling, characterising both sporadic thoracic and abdominal aneurysm, seems prevalently to be the result not only of TGF-β pathways, but also of upregulation of multiple cytokines, including interleukin-10 (IL-10), an anti-inflammatory cytokine able to modulate activity of TGF-β pathways. A large variety of immune and tissue aorta cells evocate the typical aorta abnormalities of thoracic and abdominal sporadic aneurysms. The balance of cellular type and resultant cytokine milieu determines the ultimate fate of the aortic wall healing, atherosclerosis, or aneurysm formation. In the complex scenario, another crucial factor is the genetics propensity [15, 16]. Polymorphisms of IL-10 gene have been associated with abdominal aneurysms, while no data exist in literature about their role in sporadic thoracic aneurysms [17–20].

Based on these observations, in this paper we sought to analyse the role of some common single nucleotide polymorphisms (SNPs) of genes encoding TGF-β isoforms and receptors, and IL-10 and receptor in sporadic TAA. On the other hand, no literature data on the role of genetic variants of TGF-β and IL-10 pathways in sporadic TAA exist until now.

2. Materials and Methods

2.1. Patient and Control Populations. Our study included 144 individuals (107 men (74.3%) and 37 (25.7%) women; mean age: 63 ± 10.7) from Western Sicily enrolled precisely from January 2004 to July 2008 at time of their admission to Cardiac Surgery Unit of Palermo University Hospital. They were affected by sporadic TAA, diagnosed through ECHO, CT, and MRI imaging technologies and with localization essentially in ascending aorta (precisely in aortic sinus and tubular portion and sometimes only in tubular portion) and in aortic bulb, or both (Table 1). Familial and syndromic forms (i.e., Marfan and Ehlers-Danlos syndromes) and autoimmune connective tissue disorders were excluded through histopathological criteria and phenotypic analyses.

Medical histories pertinent to aortic diseases were obtained from patient's medical records. Thus, demographic and clinical features, comorbidity conditions, and pharmacological treatments were collected (Table 1).

To perform genotype analyses two different control populations were also enrolled. The first included 90 unrelated patients of the same cardiac unit without TAA (56 (62%) men and 34 (38%) women; mean age: 61.08 ± 5.83 years). The second control group was represented by 168 healthy control (112 (66.7%) men and 56 (33.3%) women; mean age: 45.2 ± 7.44 years). Their demographic and clinical features ECHO imaging exclusion of aorta wall abnormalities, comorbidity conditions, and pharmacological treatments were collected (see Table 1).

Patients and controls belonged to the same ethnic group, since their parents and grandparents were born in Sicily. Healthy control age was significantly lower respect to that of the two groups of patients and hypertension characterised the 79% of all patients, opportunely treated with medications like ACE inhibitors and beta-blocker, and so forth during the follow-up and after surgical procedures (Table 1).

Our study received approval from local ethic committee and all participants gave their informed consent. Data were encoded to ensure privacy protection of patients and controls. All laboratory procedures were performed without knowledge about nature of material.

2.2. Molecular Typing. As reported in Table 2, we selected ten functional and common SNPs of IL-10 and TGF-β pathways located in the promoter region, codifying and noncodifying sequences and 3′UTR region. Information about these SNPs was acquired from dbSNP NCBI, the ENSEMBL database (http://www.ensembl.org/index.html), and the UCSC Genome Browser website (http://genome.ucsc.edu/). The allelic and genotypic frequencies of TGF-β and IL-10 SNP pathways were detected through the assays on demand developed by KBioscience Ltd. (Middlesex, UK) and based on a homogeneous Fluorescence Resonance Energy Transfer (FRET) detection and allele specific PCR (Kaspar). Briefly, two specific oligonucleotides were designed for each allele of the SNPs studied. Each one of these oligos was tailed with an 18 bp sequence distinct from each other. Taq polymerase, dNTPs, an internal standard dye (rhodamine X, Rox), and reverse primers were included. In addition, the KBioscience modified versions of Taq polymerase are unable to extend primers characterised to be mismatched at their 3′ terminal base. This property was used to discriminate the two alleles. The reaction was monitored by the fluorescence signals released by two other FRET reporter oligos included in the reaction mixes. The endpoint fluorescence emission was detected on an ABI-Prism 7300 Real-Time PCR Analyzer (Applied Biosystem, USA). The genotypes were determined using the 7300 system SDS software, versus 1.3 (Applied

Table 1: Demographic and clinical characteristics of TAA patients and control subjects.

Variables	AAT patients	Control patients	P	Control patients	P
Demographic characteristics	$N = 144$	$N = 90$		$N = 168$	
Age, mean (SD)	63.0 (10.7)	61.1 (5.8)	0.834	45.2 (7.4)	<0.0001
Males, number (%)	107 (74.3)	56 (62.2)	0.060	112 (66.7)	0.172
Body mass index, mean (SD)	27.0 (4.3)	26.9 (2.9)	0.898	25.8 (8.7)	0.133
TAA size and location					
Size (mm), mean (SD)	53.3 (8)	0 (0)		0 (0)	
Location, number (%)		0 (0)		0 (0)	
Ascending aorta	72 (50.0)				
Aortic bulb	16 (11.1)				
Ascending aorta and aortic bulb	56 (38.9)				
Medical history number (%)					
Aortic aneurysm familiarity	8 (5.6)	0 (0)		0 (0)	
Cardiovascular ischemic familiarity	53 (36.8)	24 (26.7)	0.089	0 (0)	
Smoking	65 (45.1)	46 (51.1)	0.420	67 (39.9)	0.360
Hypertension	114 (79.1)	28 (31.1)	<0.001	0 (0)	
Dislipidemy	33 (22.9)	14 (15.6)	0.158	0 (0)	
Diabetes mellitus	22 (15.3)	12 (13.3)	0.677	0 (0)	
Renal failure	4 (2.8)	0 (0)	0.168	0 (0)	
Dissection	16 (11.1)	0 (0)		0 (0)	
Aortic valve pathology, number (%)					
Normal	81 (56.2)	90 (100)		168 (100)	
Prolapse	19 (13.2)	0 (0)			
Vascular calcium fibrosis	45 (31.3)	0 (0)			
Aortic valve dysfunction, number (%)					
Normal	29 (20.1)	90 (100)		168 (100)	
Faint incontinence	26 (18.0)	0 (0)			
Moderate incontinence	30 (20.8)	0 (0)			
Severe incontinence	40 (27.1)	0 (0)			
Faint stenosis	1 (0.7)	0 (0)			
Moderate stenosis	2 (1.4)	0 (0)			
Severe stenosis	16 (11.1)	0 (0)			
Atherosclerosis coronary syndrome number (%)	49 (34.0)	0 (0)		0 (0)	
Drugs, number(%)					
None				168 (100)	
Beta-blockers	56 (38.9)	0 (0)		0 (0)	
Central-adrenergic agonists	23 (16.0)	0 (0)		0 (0)	
Sartans	29 (20.1)	0 (0)		0 (0)	
Calcium-channel blockers	42 (29.2)	0 (0)		0 (0)	
ACE inhibitors	59 (41.0)	14 (15.6)		0 (0)	
Antidiabetic drugs	17 (11.8)	12 (13.3)		0 (0)	
Antiaggregant drugs	46 (31.9)	28 (31.1)		0 (0)	
Antidyslipidemic drugs	32 (22.2)	0 (0)		0 (0)	
Diuretics	32 (22.2)	28 (31.1)		0 (0)	

Biosystems) sample by sample, on the basis of the detection of a unique (homozygous samples) or double (heterozygous samples) fluorescence signals.

2.3. Statistical Analysis. Allele and genotype frequencies were evaluated by gene count. Data were tested for goodness of fit between observed and expected genotype frequencies according to Hardy-Weinberg equilibrium, by χ^2 tests. Significant differences in homozygous and heterozygous genotype distributions among groups were calculated by using χ^2 test and appropriate tables. Multiple logistic regression models were applied using dominant (major allele homozygotes versus heterozygotes plus minor allele homozygotes) and recessive (major allele homozygotes plus heterozygotes versus

Table 2: Genes, SNPs (accession number), substitutions, localization, and position investigated in the study.

Genes	SNPs	Localization	Position	Alleles
IL-10	rs1800896	Promoter	−1082	G>A
	rs1800871	Promoter	−819	C>T
	rs1800872	Promoter	−592	C>A
	rs3024496	3′UTR	Not defined	C>T
IL-10RB	rs2834167	Codifying sequencing	Codon 47	A>G
TGF-β1	rs1800471	Codifying sequencing	Codon 25	G>C
	rs900	3′UTR	+94862	A>T
	rs334348	3′UTR	Not defined	A>G
	rs334349	3′UTR	Not defined	A>G
	rs4522809	Intron	+3919	C>T

minor allele homozygotes) models. Odds ratios (OR), 95% confidence intervals (95% C.I.), and P values were determined using SPSS (SPSS Inc., Chicago, IL, USA). A $P < 0.05$ was considered statistically significant.

3. Results

3.1. Analysis of the Frequencies of Ten SNPs in Our Population. Literature data have evidenced the association of the IL-10 and TGF-β SNPs with sporadic TAA and other cardiovascular diseases [4, 8, 12–14, 17–20]. The analysis of genotype frequencies of all SNPs examined with respect to the expected results was executed confirming that all populations were in Hardy-Weinberg equilibrium, with the exception of rs334349 genotype distribution (Table 3) in TAA patient group. The analysis of IL-10 and IL-10RB gene SNPs does not allow finding significant differences in genotype frequencies among the three populations examined (Table 3).

Comparing genotype distributions and allele frequencies of the five TGF-β pathway SNPs selected in our study between cases and the two control groups, significant differences were observed only for TGF-β2 rs900 polymorphism (see Table 4). Its frequency was significantly different in TAA patients than to both control patients ($P = 0.047$) and healthy controls ($P = 0.0059$) (Table 4). In particular, the AA genotype of TGF-β2 rs900 SNP had a reduced frequency in the TAA patients, which contrarily showed an increased frequency of TT genotype.

These results were confirmed by logistic regression analyses of dominant and recessive models performed between TAA patient and control groups (Table 5). Interestingly, the data obtained through comparisons between both control patients and healthy controls for dominant model and comparisons with the last group for recessive model evidenced that the presence of A allele in homo- or heterozygosis seems to be significantly protective against TAA.

Since the incidence of TAA is higher in men than in women with precisely a ratio of 3 : 1 [12], we assessed the rs900 TGF-β SNP frequencies according to gender. Comparing the data, we observed significant differences in genotype distribution of the rs900 SNP in women, whereas no significant differences were detected in men (Table 6). In particular, the AA genotype was significantly decreased in women affected by TAA with respect to both women of control patient group ($P = 0.0076$) and health control group ($P = 0.0003$). The TT genotype was reciprocally significantly increased ($P = 0.0027$). Thus, altogether these data emphasize cotemporally the gender related protective role of AA genotype for TAA and the increased susceptibility for TAA in individual's carriers of TT genotype (Table 6). When we perform a logistic regression analysis adjusted for gender, the significant differences of AA genotype frequency between patients and subjects of the two control groups ($P = 0.003$) and particularly between patients and healthy controls ($P < 0.0001$) were confirmed.

Table 3: Single nucleotide polymorphism frequencies of IL-10 pathway genes in patients affected by sporadic TAA and the two groups of controls subjects*.

SNPs	Genotypes	TAA patients n (%)	Control patients n (%)	Healthy controls n (%)
rs1800896	GG	17 (11.8)	7 (7.8)	21 (12.5)
	GA	66 (45.8)	44 (48.9)	67 (39.9)
	AA	61 (42.4)	39 (43.3)	80 (47.6)
rs1800871	CC	64 (44.4)	45 (50.0)	84 (50.0)
	CT	65 (45.1)	38 (42.2)	67 (39.9)
	TT	15 (10.5)	7 (7.8)	17 (10.1)
rs1800872	CC	64 (44.4)	45 (50.0)	84 (50.0)
	CA	65 (45.1)	38 (42.2)	67 (39.9)
	AA	15 (10.5)	7 (7.8)	17 (10.1)
rs3024496	CC	16 (11.1)	5 (5.5)	19 (11.3)
	CT	66 (45.8)	52 (57.8)	69 (41.1)
	TT	62 (43.1)	33 (36.7)	80 (47.6)
rs2834167	AA	67 (46.6)	35 (38.9)	91 (54.2)
	AG	66 (45.8)	44 (48.9)	60 (35.7)
	GG	11 (7.6)	11 (12.2)	17 (10.1)

*No significant differences were found comparing TAA patient genotype frequencies with control patient and healthy control groups.

4. Discussion

Risk factors involved in developing aneurysms are similar to those for heart disease, including atherosclerosis, hypertension, smoking, advanced age, and family history. However, the lack of aneurysm-specific symptoms often renders them unnoticed until the aorta ruptures associated with significant morbidity and mortality [1, 2, 13].

TAA development proceeds as a multifactorial process influenced by both cellular and extracellular mechanisms, resulting in alterations of structure and ECM composition [1, 2]. Recent evidence underlines the deregulation of TGF-β signalling in ascending TAAs from syndromic (Marfan

Table 4: Genotype frequencies of TGF-β1 and 2 isoform and R1 and R2 receptor gene single nucleotide polymorphisms in patients affected by sporadic TAA and the two groups of controls subjects.

SNPs	Genotypes	TAA patients *n* (%)	Control patients *n* (%)	*P* value	Healthy controls *n* (%)	*P* value
rs1800471 TGF-β1 cod25	GG	121 (84.03)	77 (85.56)	0.499	138 (82.14)	0.849
	CG	22 (15.28)	11 (12.22)		28 (16.67)	
	CC	1 (0.69)	2 (2.22)		2 (1.19)	
rs900 TGF-β2 3′UTR	AA	44 (30.56)	40 (44.44)	**0.047**	77 (45.84)	**0.0059***
	AT	70 (48.61)	36 (40.00)		73 (43.45)	
	TT	30 (20.83)	14 (15.56)		18 (10.71)	
rs334348 TGF-βR1 3′UTR	AA	92 (63.89)	56 (62.22)	0.898	106 (63.09)	0.0994
	AG	43 (29.86)	27 (30.00)		57 (33.93)	
	GG	9 (6.25)	7 (7.78)		5 (2.98)	
rs334349 TGF-βR1 3′UTR	GG	91 (63.20)	55 (61.11)	0.376	112 (66.67)	0.0544
	GA	39 (27.08)	30 (33.33)		51 (30.35)	
	AA	14 (9.72)	5 (5.56)		5 (2.98)	
rs4522809 TGF-βR2 Intron +3919	CC	36 (25.00)	24 (26.67)	0.959	44 (26.19)	0.1438
	CT	65 (45.14)	40 (44.44)		83 (49.41)	
	TT	43 (29.86)	26 (28.89)		41 (24.40)	

*The genotype distribution of rs900 TGF-β2 3′UTR SNP was significantly different in TAA patients when compared to both control patients and healthy controls. Allele frequencies: TAA patients: 0.549; control patients: 0.644; healthy subjects: 0.676.

Table 5: Multiple logistic regression analyses of dominant (major allele homozygotes versus heterozygotes plus minor allele homozygotes) and recessive (major allele homozygotes plus heterozygotes versus minor allele homozygotes) models applied to TAA patient group compared with control groups.

SNP	Model	TAA versus control patients		TAA versus healthy controls	
		OR (95% C.I.)	*P*	OR (95% C.I.)	*P*
rs1800471	Dominant	0.888 (0.452–1.858)	0.859	1.144 (0.630–2.075)	0.769
	Recessive	3.250 (0.290–36.392)	0.561	1.723 (0.154–19.210)	1.000
rs900	Dominant	0.550 (0.318–0.950)	**0.036**	0.520 (0.326–0.829)	**0.0073**
	Recessive	0.700 (0.348–1.407)	0.690	0.456 (0.242–0.859)	**0.0177**
rs334348	Dominant	1.074 (0.623–1.853)	0.889	1.035 (0.652–1.643)	0.906
	Recessive	1.265 (0.454–3.526)	0.791	0.460 (0.151–1.406)	0.181
rs334349	Dominant	1.093 (0.635–1.880)	0.782	0.858 (0.538–1.369)	0.553
	Recessive	0.546 (0.190–1.572)	0.329	0.511 (0.081–1.039)	0.0516
rs4522809	Dominant	0.917 (0.503–1.671)	0.878	0.939 (0.564–1.565)	0.897
	Recessive	0.954 (0.535–1.703)	1.000	0.758 (0.459–1.252)	0.307

syndrome, Loeys-Dietz syndrome, and Ehlers-Danlos syndrome) and not syndromic TAA patients as well as in TAAs from cases affected by familial TAAs and dissections [14].

TGF-β isoforms are produced by multiple cellular types and participate in a wide array of cellular responses including proliferation, angiogenesis, differentiation, apoptosis, inflammation, and wound healing [21]. Of the three TGF-β isoforms, their role in matrix deposition (e.g., collagen synthesis) related to fibrotic disease is particularly well known [22]. However, some recent data have also demonstrated their involvement in unconventional pathways able to determine matrix degradation [4].

Furthermore, the TGF-β isoforms exhibit both overlapping and divergent properties, as evidenced principally in embryogenetic studies. In particular, TGF-β2 knockout mice are characterized to die perinatally and display a wide range of developmental defects, including cardiovascular, pulmonary, skeletal, ocular, inner ear, and urogenital manifestations [23–25]. Haplo-insufficient TGF-β2 mice have aortic root aneurysm and biochemical evidence of increased canonical and no canonical TGF-β signalling [26]. These observations led Maleszewska and colleagues to suggest the crucial role of TGF-β2 in the vascular remodelling [27]. They particularly underlined that, in presence of a low grade chronic inflammation of the cardiac and vascular tissues, the TGF-β2, interacting with other cytokines as interleukin-1B, might modify and remodel vascular aorta tissues inducing endothelial cells forward mesenchymal transition (EndMT). EndMT represents a central mechanism in cardiac valve embryogenesis, which in pathological condition might determine cardiac fibrosis.

Table 6: Statistical analysis of TGF-β2 rs900 genotype distributions in patients affected by TAA and in the two groups of control subjects stratified according to the gender*.

	Men			Women		
	AA	AT	TT	AA	AT	TT
TAA patients *n* (%)	36 (33.65)	54 (50.46)	17 (15.89)	8 (21.62)	16 (43.24)	13 (35.13)
Control patients *n* (%)	22 (39.29)	25 (44.64)	9 (16.07)	18 (52.94)	11 (32.35)	5 (14.71)
Healthy controls *n* (%)	43 (38.39)	56 (50.00)	13 (11.61)	34 (60.71)	17 (30.36)	5 (8.93)
	Odd ratio significance					
	Men			Women		
	AA	AT	TT	AA	AT	TT
	TAA versus control patients					
OR	0.784	1.263	0.986	0.245	1.263	3.142
95% C.I.	0.401–1.531	0.660–2.418	0.408–2.382	0.087–0.689	0.660–2.418	0.990–10.071
P value	0.495	0.512	1.000	**0.0076**	0.512	0.059
	TAA versus healthy controls					
OR	0.814	1.019	1.438	0.178	1.019	5.525
95% C.I.	0.468–1.415	0.401–1.531	0.662–3.128	0.069–0.461	0.600–1.731	1.767–17.272
P value	0.484	1.000	0.433	**0.0003**	1.000	**0.0027**

*Logistic regression analysis adjusted for gender confirms that the AA genotype frequency was significantly decreased and TT genotype increased in TAA women patients compared to women of the two control groups ($P = 0.003$), in particular compared to healthy controls ($P < 0.0001$).

Actually, immunohistochemistry analysis of TAA aneurysms demonstrated that both the media and adventitia from patients with Marfan syndrome and familial TAAs, as well as from sporadic cases with or without dissections or BAV diseases, are characterised by infiltration of inflammatory cells [27]. This inflammatory condition might contribute to the pathogenesis of TAA [3, 28–35].

In the light of these observations, we assessed the role of five genetic variants of TGF-β pathways (TGF-β1 and 2 isoforms and R1 and R2 receptors) in sporadic TAA. Interestingly, the most relevant finding of the present study allows proposing that rs900 TGF-β2 SNP is associated with sporadic TAA in women. On the other hand, recent reports assigned a direct or an indirect central role to TGF-β2 and its genetic variants in the pathogenesis of both syndromic and familial TAAs [3, 21, 28–35]. In addition, it has been reported that mutations in the TGFBRII genes deregulate the TGF-β2 signalling pathway involved in TAA pathogenesis [3, 28–35]. TGF-β2 gene mutations have been found in familial TAAs and dissections associated with mild systemic features of Marfan syndrome, Loeys-Dietz syndrome and in TAA and dissection associated with mitral valve disease [3, 28–35]. In these diseases, the TGF-β2 dependent EndMT might play a role. In spite of these findings, the exact role of TGF-β2 in TAA pathogenesis is not clear. In particular, both the loss of function genetically determined and the "paradoxical" augment in the downstream TGF-β signaling pathway might be important for TTA development [14].

However, to our knowledge, no literature data exist about the role of genetic variants of TGF-β2 pathway in sporadic TAAs. Thus, this is the first report that identified a common and functional TGF-β2 SNP, the rs900 SNP, as genetic risk marker for sporadic TAA. The rs900 SNP is located at the 2814 position downstream of the TGF-β2 gene coding region. Scanning allelic rs900 sequences for UTR structural motif using an online tool (http://itbtools.ba.itb.cnr.it/utrscan) has shown that the T allele introduces a new open reading frame ATG in the 3′UTR region of TGF-β2 gene that potentially might interfere with ribosomal translation. This may allow hypothesising that the rs900 T allele may interfere with the rate of protein production.

5. Limitations and Conclusions

As reported above, TAAs occur most frequently in Caucasians than in other ethnic groups and they afflict men two to four times more frequently than women [4]. As consequence, our results, suggesting that rs900 TGF-β2 SNP might be one of genetic factors involved in the woman susceptibility for TAA, might open new perspectives for the analysis of sporadic TAA susceptibility factors and prevention. Actually these findings obtained in this relatively small study, which need certainly to be confirmed in larger populations of different genetic background, might prompt studies on gender oriented pharmacological strategies to prevent TAA development in predisposed subjects.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Statin Treatment Is Associated with Reduction in Serum Levels of Receptor Activator of NF-κB Ligand and Neutrophil Activation in Patients with Severe Carotid Stenosis

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Systemic and intraplaque biomarkers have been widely investigated in clinical cohorts as promising surrogate parameters of cardiovascular vulnerability. In this pilot study, we investigated if systemic and intraplaque levels of calcification biomarkers were affected by treatment with a statin in a cohort of patients with severe carotid stenosis and being asymptomatic for ischemic stroke. Patients on statin therapy had reduced serum osteopontin (OPN), RANKL/osteoprotegerin (OPG) ratio, and MMP-9/pro-MMP-9 activity as compared to untreated patients. Statin-treated patients exhibited increased levels of collagen and reduced neutrophil infiltration in downstream portions of carotid plaques as compared to untreated controls. In upstream plaque portions, OPG content was increased in statin-treated patients as compared to controls. Other histological parameters (such as lipid, macrophage, smooth muscle cell, and MMP-9 content) as well as RANKL, RANK, and OPG mRNA levels did not differ between the two patient groups. Serum RANKL/OPG ratio positively correlated with serum levels of neutrophilic products, intraplaque neutrophil, and MMP-9 content within downstream portions of carotid plaques. In conclusion, statin treatment was associated with improvement in serum RANKL levels and reduced neutrophil activity both systemically and in atherosclerotic plaques.

1. Introduction

Atherosclerotic plaque calcification is considered a common process in advanced atherogenesis, although its association with increased plaque vulnerability remains unclear. On the one hand, the coronary calcium score (CAC) was shown to improve the Framingham risk score prediction in intermediate risk individuals [1] and to be an independent predictor of stroke in subjects deemed at low risk (accordingly to clinically based traditional risk stratifications tools) [2]. On the other hand, histological findings from human carotid atherosclerotic plaques demonstrated that symptomatic atheroma is characterized by reduced levels of calcification and increased inflammation as compared to

asymptomatic plaques [3]. This scenario is further complicated by recent recommendations that "intraplaque vulnerability" is just considered as one of multiple determinants of cardiovascular risk (i.e., intraplaque, circulating, and peripheral tissue risk factors), suggesting the importance of defining systemic risk biomarkers and of understanding their pathophysiological role [4, 5]. Potential "systemic" biomarkers of intraplaque calcification include osteopontin (OPN), osteoprotegerin (OPG), and receptor activator of nuclear factor kB ligand (RANKL) [6–8]. In this context, we have recently shown that RANKL promotes the degranulation liberating proatherosclerotic products by neutrophils [9]. Notably, specific anti-RANKL neutralizing antibodies for clinical applications (i.e., denosumab) have been developed and are currently investigated as a way to block skeletal bone resorption in cancer diseases [10]. Since cardiovascular outcomes were not considered as endpoints in available cancer-related trials [11], the potential effects of those therapeutic compounds on atherogenesis are still elusive, and knowing whether conventional antiatherosclerotic treatments could affect RANKL signalling remains an open question.

Therefore, in this work, we studied the potential anti-RANKL effects of the 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors (statins) in patients with severe carotid plaque stenosis and being asymptomatic for acute ischemic diseases.

Statins are the most common lipid-lowering drugs worldwide and are recommended for the prevention of cardiovascular diseases [12]. Their antiatherosclerotic effects include not only lipid profile improvements, but also additional "pleiotropic" activities (such as anti-inflammatory, antioxidative, and immunomodulatory activities), which all contribute to stabilizing atherosclerotic plaques [13, 14] and reduction of systemic atherosclerotic factors, such as C-reactive protein (CRP) [15]. For these reasons, statins might be very promising candidates to potentially reduce systemic and intraplaque RANKL levels and/or bioactivity (as a neutrophil activator) in advanced atherosclerosis.

2. Methods

2.1. Patients and Study Design. We conducted an observational cohort study between December 2010 and November 2011 at a single hospital (San Martino Hospital) in Genoa (Italy). Patients ($n = 38$) undergoing carotid endarterectomy (CEA) for extra cranial high-grade internal carotid stenosis (>70% luminal narrowing) [4–6] and being asymptomatic for ischemic stroke were included in the study. Patients were defined as asymptomatic when they had no history of ischemic symptoms and in the absence of signs of cerebral necrosis at magnetic resonance imaging (MRI) with diffusion sequences. The exclusion criteria were malignant hypertension, acute coronary artery disease, any cardiac arrhythmias, congestive heart failure (II, III, and IV NHYA classes), liver or renal disorders or function abnormalities, acute and chronic infectious disease, autoimmune and rheumatic diseases, osteoporosis, cancer, endocrine diseases, inflammatory bowel diseases and anti-inflammatory (other than aspirin) medications, oral anticoagulant treatments, hormone, and cytokine or growth factor therapies.

The day before endarterectomy, blood samples were obtained by peripheral venipuncture from these patients at fasting state to collect serum and plasma and to assess blood parameters. Medications reported in Table 1 were not modified in the 2 months prior to enrolment. Within the cohort, patients under treatment with a statin ($n = 26$) were compared with patients without statins ($n = 12$) for both systemic and intraplaque inflammatory and vascular parameters. Different statins and doses taken by patients were shown in Table 2. The Medical Ethics Committee of San Martino Hospital approved the study, and participants provided written informed consent. The study was conducted in compliance with the Declaration of Helsinki.

2.2. Power Estimation. Power calculation was based on prior published literature [16, 17]. Our sample size (26 patients under statins versus 12 patients without statin) allowed us to detect large effect sizes (>0.80) for systemic inflammatory markers or intraplaque parameters between symptomatic and asymptomatic patients with a power of 80%, taking a two-sided type I error of 5%.

2.3. Systemic Inflammatory Marker Detection. Serum C-reactive protein (CRP), osteopontin (OPN), osteoprotegerin (OPG), myeloperoxidase (MPO), matrix metalloproteinase (MMP)-8, MMP-9, tissue inhibitor of metalloproteinase (TIMP)-1, TIMP-2, TIMP-3, TIMP-4, and MMP9/TIMP-1 levels were measured by colorimetric enzyme-linked immunosorbent assay (ELISA, from R&D Systems, Minneapolis, Minnesota), following manufacturer's instructions. Serum RANKL levels were measured by ELISA (BioVendor GmbH, Heidelberg, Germany), following manufacturer's instructions. Serum neutrophil elastase levels were assessed by ELISA (Bender Med Systems GmbH, Vienna, Austria), following manufacturer's instructions. The limits of detection were 0.78 ng/mL for CRP, 0.312 ng/mL for OPN, 0.0625 ng/mL for OPG, 0.156 ng/mL for MPO, 0.156 ng/mL for MMP-8, 0.312 ng/mL for MMP-9, 0.156 ng/mL for TIMP-1, 0.156 ng/mL for TIMP-2, 0.0625 ng/mL for TIMP-3, 0.0781 ng/mL for TIMP-4, 0.0469 ng/mL for MMP/TIMP-1, 31.25 pg/mL for RANKL, and 0.156 ng/mL for neutrophil elastase. Mean intra- and interassay coefficients of variation (CV) were below 8% for all markers. Glucose, triglycerides, total cholesterol, low-density lipoprotein (LDL) cholesterol, and high-density lipoprotein (HDL) cholesterol were routinely measured and expressed in mg/dL. Serum insulin and C-peptide were routinely measured and expressed in mU/L and μg/L, respectively.

2.4. Pro-MMP-9 Zymographic Assay. Pro-MMP-9 zymographic activity was assessed in human serum, as previously described [18]. 9% of SDS-polyacrylamide gels were copolymerized with gelatin (Sigma, St. Louis, MO). Equal amounts of patient serum (2 μL, stored at −80°C) and 1 ng of recombinant pro-MMP-9 standard (Calbiochem, Lucerne, Switzerland) were loaded on gels in the absence of reducing

Table 1: Clinical characteristics and medications of the study population.

Characteristics	Statin ($n = 26$)	No statin ($n = 12$)	P value
Age, yr (IQR)	72.0 (66.0–77.3)	71.5 (67.3–76.7)	0.9249
Males, n (%)	14 (53.8)	7 (58.3)	1.0000
CV risk factors			
Blood pressure, mmHg			
Systolic (IQR)	130.0 (125.0–141.3)	130.0 (125.0–140.0)	0.6826
Diastolic (IQR)	85.0 (80.0–90.0)	80.5 (80.0–84.5)	0.4686
Waist Circumference, cm (IQR)	91.0 (88.0–98.0)	91.5 (90.0–95.0)	0.7414
Current smoking, n (%)	7 (26.9)	3 (25.0)	1.0000
Type 2 diabetes, n (%)	11 (42.3)	3 (25.0)	0.4722
Dislipidemia, n (%)	16 (61.5)	4 (33.3)	0.1643
Hypertension, n (%)	21 (80.8)	10 (83.3)	1.0000
Chronic CAD*, n (%)	7 (26.9)	3 (25)	1.0000
Carotid stenosis % lumen (IQR)	75 (70–80)	80 (70–85)	0.2349
Biological parameters			
Total WBC†, number $\times 10^9$/L (IQR)	6.35 (5.53–7.43)	6.56 (5.60–8.98)	0.4053
Neutrophils, number $\times 10^9$/L (IQR)	3.90 (3.38–5.05)	4.36 (3.57–6.14)	0.4996
Lymphocytes, number $\times 10^9$/L (IQR)	1.58 (1.24–1.96)	1.56 (1.12–1.84)	0.6716
Monocytes, number $\times 10^9$/L (IQR)	0.37 (0.30–0.49)	0.44 (0.38–0.53)	0.0429
Red blood cells, number $\times 10^{12}$/L (IQR)	4.60 (4.10–4.85)	4.65 (4.53–5.00)	0.2513
Platelet, number $\times 10^9$/L (IQR)	207.5 (178.8–238.8)	188.5 (175.5–219.8)	0.5096
Plasma fibrinogen, g/L (IQR)	3.65 (3.25–4.79)	4.09 (3.69–5.11)	0.3146
Serum total-c‡, mg/dl (IQR)	189.0 (164.0–215.0)	202.0 (159.3–209.3)	0.6150
Serum LDL-c§, mg/dl (IQR)	104.0 (81.2–130.0)	118.8 (77.0–132.2)	0.6380
Serum HDL-c‖, mg/dl (IQR)	60.0 (41.5–69.0)	49.5 (41.5–64.0)	0.4754
Serum triglycerides, mg/dl (IQR)	124.0 (83.5–160.0)	85.0 (68.0–118.8)	0.2237
Serum glycaemia, mg/dl (IQR)	101.0 (96.5–153.0)	111.0 (95.8–124.0)	0.6497
Serum insulinemia, mU/L (IQR)	7.5 (5.8–16.3)	5.6 (3.7–12.2)	0.0747
Serum C-peptidemia, μg/L (IQR)	2.35 (1.92–3.97)	2.06 (1.60–2.61)	0.1894
Antiplatelets, n (%)	23 (88.5)	10 (83.3)	0.6426
Diuretics, n (%)	2 (7.7)	4 (33.3)	0.0661
ACE# inhibitors, n (%)	2 (7.7)	1 (8.3)	0.2295
ARBs**, n (%)	11 (42.3)	6 (50.0)	0.7342
Beta-blockers, n (%)	10 (38.5)	3 (25.0)	0.4859
Calcium channel blockers, n (%)	9 (34.6)	4 (33.3)	1.0000
Oral antidiabetics, n (%)	7 (26.9)	3 (25.0)	1.0000

Continuous variables are expressed as median (interquartile range (IQR)).
*CAD: coronary artery disease.
†WBC: white blood cells.
‡total-c: total cholesterol.
§LDL-c: low-density lipoprotein cholesterol.
‖HDL-c: high-density lipoprotein cholesterol.
#ACE: angiotensin converting enzyme.
**ARBs: angiotensin receptor blockers.

agents. Then, gels were rinsed and stained with Coomassie Blue R-250. Zymographic results were expressed as pro-MMP-9 proteolytic activity and calculated on the basis of the following formula: Serum pro-MMP-9 = $(I_{obs}/I_{std}) \times W_{std}$, where I_{obs} and I_{std} are intensities of lytic areas produced in gels by samples and by standard pro-MMP-9 and W_{std} is the weight (1 ng) of standard pro-MMP-9 loaded onto the gel. Zymographic data were expressed as ng/mL of serum. Gelatinolytic bands were measured with a gel analysis system (GeneGenius, Syngene, Cambridge, UK).

2.5. Human Carotid Plaque Specimen Processing. Shortly after surgical excision, the internal carotid plaque specimens were taken from all patients and immediately transferred at 4°C to the laboratory for processing. Carotid plaques had the morphology of calcified advanced lesions with a lipid-rich

Table 2: Different statin treatments.

Statin (dose)	*n* (%)
Rosuvastatin (5 mg/day)	12 (46.15)
Simvastatin (40 mg/day)	6 (23.08)
Atorvastatin (20 mg/day)	8 (30.77)

acellular core. The internal carotid plaque specimens were cut perpendicularly to the long axis through the point of maximum stenosis to obtain two portions (upstream and downstream blood flow) [18]. Each portion was further divided perpendicularly to the long axis in the middle into two subsegments. One half was snap-frozen in liquid nitrogen and stored at −80°C for mRNA isolation, and the other half was frozen in cryoembedding medium for histological analysis.

2.6. Immunohistochemistry. Frozen upstream and downstream human carotid specimens were serially cut into eight 7 μm sections per each portion separated by 105 μm from each other [18]. Sections were fixed in acetone and immunostained with specific antibodies, anti-human smooth muscle actin (dilution: 1 : 100; Dako Corporation, Glostrup, Denmark), anti-human CD68 (dilution: 1 : 100; Dako Corporation), anti-human CD66b (dilution: 1 : 50; Beckman Coulter, Nyon, CH), anti-human matrix metalloproteinase (MMP)-9 (dilution: 1 : 250; Sothern Biotech, Birmingham, AL), anti-human OPG (dilution: 1 : 20; R&D Systems), and anti-human RANKL (dilution: 1 : 20; R&D Systems). Quantifications were performed with MetaMorph software. Results for these parameters were calculated and expressed as percentages of stained area on total lesion area or number of infiltrating cells on mm^2 of lesion area.

2.7. Oil Red O Staining for Lipid Content. Eight sections per portion (upstream and downstream blood flow) of human carotid plaques were stained with Oil Red O, as previously described [18]. Sections were counterstained with Mayer's hemalum and rinsed in distilled water. Quantifications were performed with MetaMorph software. Data were calculated as ratios of stained area on total lesion area.

2.8. Sirius Red Staining for Collagen Content. Eight sections per portion (upstream and downstream blood flow) of human carotid plaques were rinsed with water and incubated with 0.1% Sirius red (Sigma Chemical Co, St Louis, MO) in saturated picric acid for 90 min. Sections were rinsed twice with 0.01 N HCl for 1 min and then immersed in water. After dehydration with ethanol for 30 seconds and cover-slipping, the sections were photographed with identical exposure settings under ordinary polychromatic or polarized light microscopy. Total collagen content was evaluated under polychromatic light. Interstitial collagen subtypes were evaluated using polarized light illumination; under this condition thicker type I collagen fibers appeared orange or red, whereas thinner type III collagen fibers were yellow or green [16]. Quantifications were performed with MetaMorph software. Data were calculated as percentages of stained area on total lesion area.

2.9. Real-Time RT-PCR. Total mRNA was isolated with Tri Reagent (MRC Inc.) from upstream or downstream specimens of human carotid plaques. Reverse transcription was performed using the ImProm-II Reverse Transcription System (Promega, Madison, WI) according to the manufacturer's instructions. Real-time PCR (StepOnePlus, Applied Biosystems) was performed with the ABsolute QPCR Mix (ABgene).

Specific primers and probes (Table 3) were used to determine the mRNA expression of RANK, RANKL, OPG, and RPS13 (housekeeping gene). The fold change of mRNA levels was calculated by the comparative C_t method. The measured C_t values were first normalized to the RPS13 internal control, by calculating delta C_t (ΔC_t). This was achieved by subtracting the RPS13 C_t values from the gene of interest C_t value. Delta delta C_t ($\Delta\Delta C_t$) was calculated by subtracting the designated baseline control group ΔC_t value from the study group ΔC_t values. The $\Delta\Delta C_t$ was then plotted as a relative fold change with the following formula: $2^{-\Delta\Delta C_t}$.

2.10. Statistical Analysis. Patient characteristics were described one day before endarterectomy. Patients asymptomatic for ischemic stroke and on statin treatment were compared to patients without statins using the Pearson's chi-square test or the Fisher's exact test (when appropriate) for the comparison of qualitative variables. The Mann-Whitney nonparametric test was used for comparisons of continuous variables. Results were expressed as medians (interquartile range (IQR)). Spearman's rank correlation coefficients were used to assess correlations between serum RANKL/OPG ratio and levels of serum neutrophil products and intraplaque parameters in both upstream and downstream regions of carotid atherosclerotic plaques, respectively. Values of $P < 0.05$ (two-tailed) were considered significant. All analyses were done with GraphPad Instat software version 3.05 (GraphPad Software).

3. Results

3.1. Baseline Population Characteristics. Clinical features, laboratory parameters, and medications of our cohort of asymptomatic patients with severe carotid stenosis ($n = 38$) are shown in Table 1. There was no significant difference between patients under or without treatment with a statin in terms of age, sex, classical cardiovascular risk factors, hematological parameters, and medications except for a slight but significant ($P = 0.0429$) decrease on circulating monocyte count in statin-treated group. Table 2 summarizes the statins used by the patients and their dosage. Only treatments with low doses of statins [19] were investigated in the present study (Table 2).

3.2. Statin Treatment Is Associated with the Modulation of Serum Levels of Calcification Biomarkers and MMP-9. The first goal of this study was to determine if statin treatment

Table 3: Human primers used for real-time PCR.

Gene	Function	Nucleotide sequence	Size (bp)	Accession number
RPS13	Fw	5′-CGTCCCCACTTGGTTGAAG-3′	90	NM_001017
	Rv	5′-CCGATCTGTGAAGGAGTAAGG-3′		
	Probe	5′-ACATCTGACGACGTGAAGGAGCAGATT-3′		
TNFSF11 (RANKL)	Fw	5′-AAGGAGCTGTGCAAAAGGAA-3′	75	NM_003701
	Rv	5′-CATCCACCATCGCTTTCTCT-3′		
	Probe	5′-CGTTGGATCACAGCACATCAGAGC-3′		
TNFRSF11A (RANK)	Fw	5′-CAGCTAATTTGTGGCACTGG-3′	68	NM_003839
	Rv	5′-ACCTGAGGACTCCTTATCTCCA-3′		
	Probe	5′-CAATGAGGCTTGTGGCCGCCTA-3′		
TNFRSF11B (OPG)	Fw	5′-TGGAATAGATGTTACCCTGTGTG-3′	149	NM_002546
	Rv	5′-TGTGTTGCCGTTTTATCCTCT-3′		
	Probe	5′-AGGCATTCTTCAGGTTTGCTGTTCC-3′		

Table 4: Serum cardiovascular risk markers.

Characteristics	Statin ($n = 26$)	No statin ($n = 12$)	P-value
CRP*, mg/L	2.4 (0.96–4.03)	4.1 (1.43–8.41)	0.1782
OPN, ng/mL	17.5 (12.8–23.8)	29.4 (23.2–34.6)	0.0131
RANKL, pg/mL	1022 (247–1669)	1839 (1551–2803)	0.0050
OPG, pg/mL	167.8 (62.3–318.1)	62.3 (62.3–432.0)	0.0182
RANKL/OPG ratio	5.8 (1.5–17.1)	27.2 (15.5–43.3)	0.0004
MPO, ng/mL	265.2 (101.8–531.6)	466.0 (313.7–841.9)	0.1014
Neutrophil elastase, ng/mL	211.1 (109.1–318.6)	325.3 (143.1–450.2)	0.1489
MMP†-8, ng/mL	16.5 (5.8–23.7)	12.7 (4.0–24.7)	0.7579
MMP-9, ng/mL	231.5 (134.9–431.6)	479.0 (352.8–812.5)	0.0461
Pro-MMP-9 activity, ng/mL	6.8 (3.1–14.4)	13.1 (11.9–18.2)	0.0310
MMP9/TIMP-1, ng/mL	10.6 (6.4–18.4)	14.4 (2.8–23.0)	0.8839
TIMP‡-1, ng/mL	191.3 (120.0–216.7)	211.0 (147.6–264.6)	0.3389
TIMP-2, ng/mL	101.4 (65.4–124.5)	97.0 (73.0–112.2)	0.8078
TIMP-3, ng/mL	8.1 (4.7–14.9)	12.5 (6.2–13.8)	0.5824
TIMP-4, ng/mL	5.4 (3.6–6.6)	4.2 (4.0–6.1)	0.7579

Data are expressed as median (interquartile range (IQR)).
*CRP: C-reactive protein.
†MMP: matrix metalloproteinase.
‡TIMP: tissue inhibitor of metalloproteinase.

was associated with improvements in the systemic levels of cardiovascular risk factors, calcification biomarkers, and neutrophil degranulation products, with CRP being the first biomarker to be analyzed. Statin-treated patients had similar CRP serum levels as compared to statin-untreated controls (Table 4). With respect to calcification biomarkers, serum levels of OPN and RANKL were significantly reduced, while OPG was increased in statin-treated patients as compared to patients without statins. In line with the observed changes in RANKL and OPG levels, the RANKL/OPG ratio (indicating the circulating RANKL free fraction) was significantly decreased in statin-treated patients as compared to controls. With respect to neutrophil degranulation products, we did not find any difference between the two patient groups in terms of serum MPO, neutrophil elastase, and MMP-8 levels and of protective tissue inhibitor of metalloproteinases (TIMP-1, -2, -3, and -4) (Table 4). However, the MMP-9 level and pro-MMP-9 activity were significantly reduced in patients receiving statin therapy as compared to those without statins (Table 4).

3.3. Statin Treatment Is Associated with Reduction in Neutrophil Infiltrates and Vulnerability in Carotid Plaque Portions Downstream Blood Flow. Taking into account the elevated heterogeneity of human carotid plaques [17, 18, 20], the effect of statin treatment on intraplaque inflammatory patterns and calcification biomarkers was assessed in different regions (upstream and downstream blood flow, resp.). In upstream portions, no differences were found between the two patient groups in terms of lipid and collagen content and of cell composition (SMCs, macrophages, neutrophils, and MMP-9) (Table 5, Figures 1 and 2). In downstream regions,

Table 5: Parameters of intraplaque vulnerability.

Characteristics	Statin ($n = 26$)	No statin ($n = 12$)	P-value
Upstream portion			
% of lipid	4.63 (1.84–8.63)	4.36 (3.17–5.54)	0.6524
% of total collagen	41.24 (18.91–52.70)	30.43 (21.25–43.09)	0.6786
% of collagen I	8.97 (4.27–15.98)	6.42 (4.71–10.04)	0.4385
% of collagen III	21.79 (15.32–37.75)	20.33 (16.67–31.94)	0.9569
% of smooth muscle cell-rich area	8.85 (3.75–19.57)	8.00 (3.22–14.48)	0.6013
% of macrophage-rich area	2.96 (0.88–9.06)	2.08 (0.27–6.69)	0.4823
Neutrophils/mm^2	0.75 (0.52–1.32)	0.81 (0.30–12.47)	0.6786
% of MMP* -9	0.71 (0.33–1.36)	0.39 (0.21–7.19)	0.6524
RANK† mRNA, fold increase	0.49 (0.33–1.15)	0.81 (0.31–1.74)	0.5725
RANKL‡ mRNA, fold increase	0.78 (0.30–1.24)	0.61 (0.32–0.98)	0.8859
OPG§ mRNA, fold increase	0.84 (0.57–1.47)	0.83 (0.50–1.17)	0.7944
% of OPG	2.71 (1.77–6.06)	0.50 (0.10–2.34)	0.0150
Downstream portion			
% of lipid	4.39 (2.83–8.69)	4.94 (2.13–6.63)	0.4884
% of total collagen	18.45 (2.07–45.87)	5.06 (0.50–10.28)	0.0289
% of collagen I	5.56 (0.67–20.74)	0.67 (0.11–3.37)	0.0345
% of collagen III	10.61 (2.15–25.11)	1.98 (0.70–8.57)	0.0728
% of smooth muscle cell-rich area	3.87 (1.50–5.15)	3.59 (2.42–4.30)	0.8173
% of macrophage-rich area	1.97 (1.02–8.29)	9.00 (2.24–11.70)	0.1060
Neutrophils/mm^2	0.26 (0.15–0.93)	3.77 (1.20–8.16)	0.0001
% of MMP-9	4.05 (0.68–12.30)	10.12 (3.86–13.81)	0.0574
RANK mRNA, fold increase	0.99 (0.48–2.77)	0.62 (0.38–2.43)	0.4601
RANKL mRNA, fold increase	0.84 (0.45–1.25)	0.59 (0.17–1.60)	0.5165
OPG mRNA, fold increase	1.38 (1.06–1.71)	1.06 (0.80–1.60)	0.2117
% of OPG	0.27 (0.01–0.96)	0.28 (0.02–0.67)	0.7746

Data are expressed as median (interquartile range (IQR)).
*MMP: matrix metalloproteinase.
†RANK: receptor activator of nuclear factor-κB.
‡RANKL: receptor activator of nuclear factor-κB ligand.
§OPG: osteoprotegerin.

the total collagen and collagen I contents were significantly higher in the statin-treated group as compared to the group with no statin use. Notably, this effect was associated with a virtual abrogation of intraplaque neutrophil infiltration in patients treated with statins as compared to untreated patients. Despite being close to significance ($P = 0.0574$), a similar trend in intraplaque content of the neutrophilic product MMP-9 was also observed (Table 5). No difference between the two patient groups was found in terms of intraplaque lipids and of infiltration by other cell subsets, such as SMCs and macrophages (Table 5).

Calcification biomarkers have previously been reported to be expressed within atherosclerotic plaques [21]. Thus, we focused on the expression of these markers in our cohort of patients both at mRNA and protein level. RANKL was almost undetectable in both groups at both mRNA (upstream plaques: detectable in 14 statin and 10 no statin; downstream plaques: in 17 statin and 11 no statin) and protein levels (detectable in 4 downstream portions of carotid plaques from statin-treated patients, data not shown). Statin treatment was not associated with an altered expression of RANKL, RANK (RANKL's receptor), and OPG mRNA as compared to control patients without statins (Table 5). At the protein level, intraplaque OPG expression was weak in both groups (Table 5). A significant increase in OPG protein levels was shown in upstream regions of carotid plaques from statin-treated versus untreated patients, whereas no statistically significant difference in downstream plaque portions could be detected (Table 5). Finally, the expression pattern of OPG in carotid plaques suggested that this protein did not colocalize with macrophages or neutrophils but rather with intimal SMCs and lipids (Figures 1 and 2). This result was similar in both patients' groups (Figures 1 and 2).

3.4. RANKL/OPG Ratio Positively Correlates with Neutrophil Degranulation Products Both in the Systemic Circulation and within Carotid Plaques. Spearman rank correlations were performed in order to identify potential associations between free serum RANKL levels (RANKL/OPG ratio) on the one

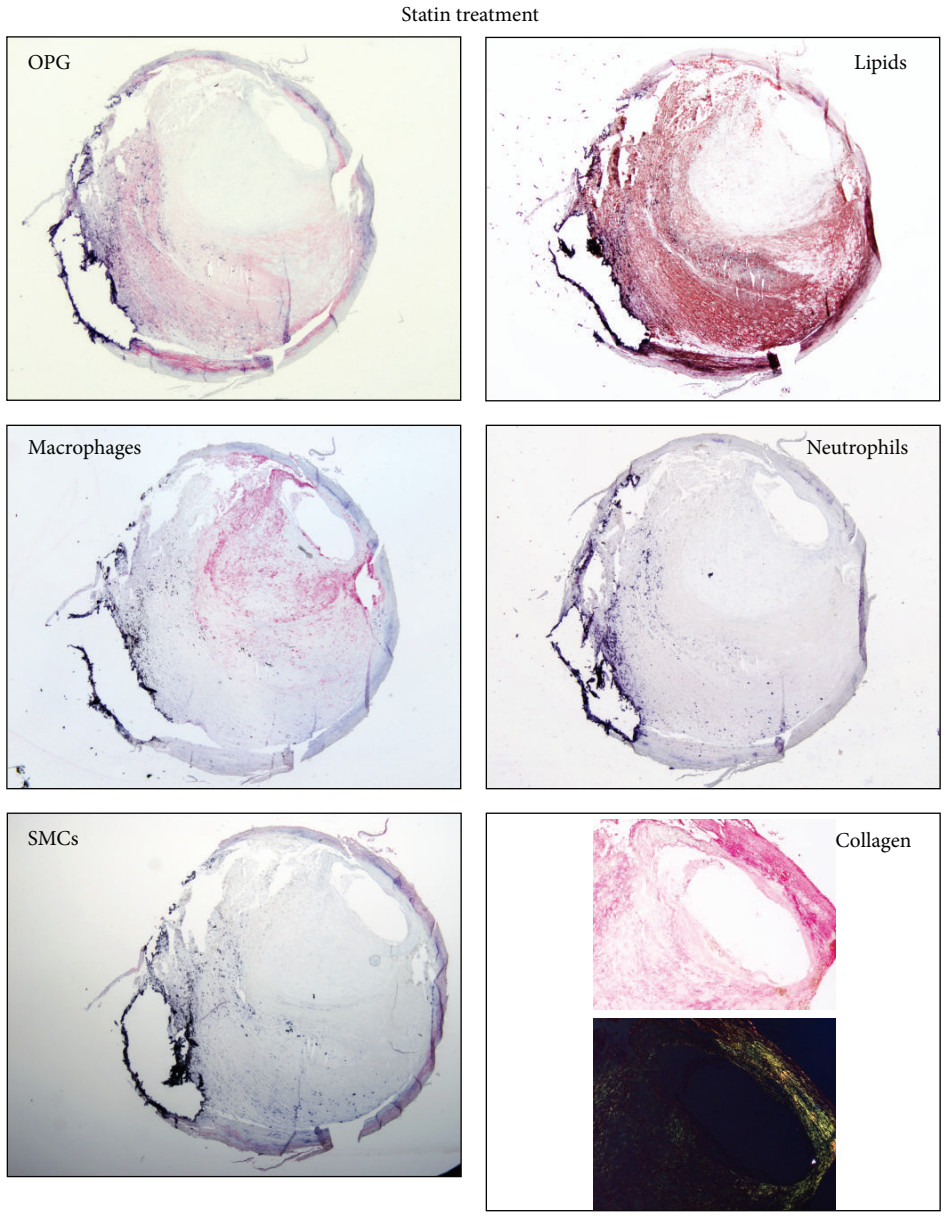

Figure 1: Representative microphotographs of consecutive cryosections from upstream regions of carotid plaques from patients on statin treatment. Staining for osteoprotegerin (OPG), lipids, macrophages, neutrophils and smooth muscle cells (SMCs), and collagen is shown.

hand and systemic and intraplaque neutrophil products on the other in our two groups of patients (statin treatment versus no statin treatment). RANKL/OPG ratio was found to positively correlate with serum CRP, MPO, and neutrophil elastase levels, as well as with serum pro-MMP-9 activity (Table 6). Though not statistically significant ($P = 0.0614$), a positive association between RANKL/OPG ratio and MMP-9 serum levels was observed (Table 6). No significant association between RANKL/OPG ratio and serum MMP-8 was detected (Table 6). Serum RANKL/OPG ratio was also shown to inversely correlate with intraplaque lipid content in upstream regions of carotid plaques and to positively correlate with neutrophil and MMP-9 content in downstream portions. No additional significant correlations were shown for other intraplaque parameters, such as collagen content, SMCs, and macrophages, in the different intraplaque regions (Table 6).

4. Discussion

The main result of this study is represented by the finding that an ongoing treatment with statins is associated with alterations in serum levels of biomarkers of plaque calcification such as OPN, RANKL, and OPG in asymptomatic patients with a severe carotid stenosis undergoing a carotid endarterectomy. In particular, the free circulating fraction of RANKL (not bound to OPG, expressed as RANKL/OPG ratio) was strongly reduced in statin-treated patients as

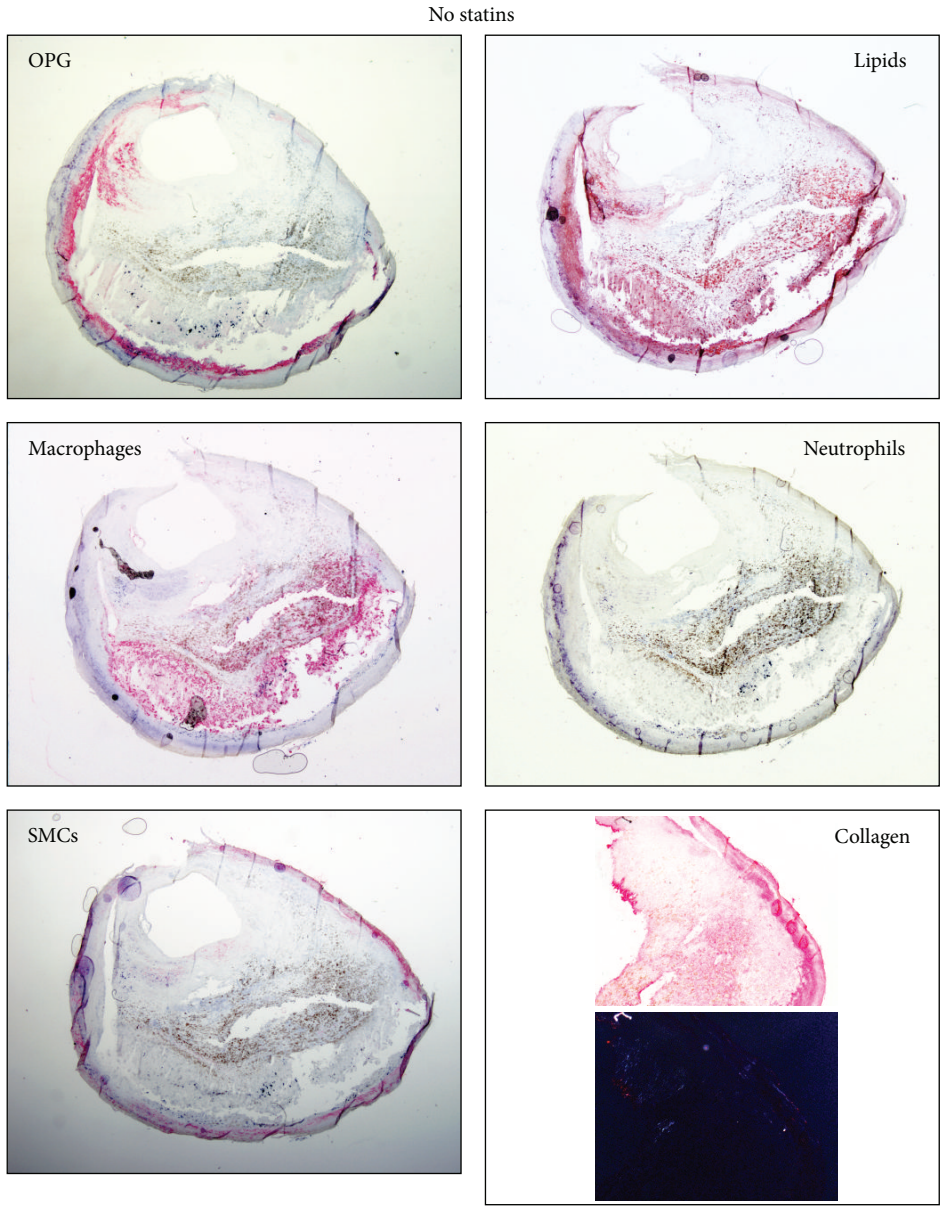

Figure 2: Representative microphotographs of consecutive cryosections from upstream regions of carotid plaques from patients without statins. Staining for osteoprotegerin (OPG), lipids, macrophages, neutrophils and smooth muscle cells (SMCs), and collagen is shown.

compared to controls without a statin. Conversely, we did not detect any relevant change in intraplaque RANKL/OPG levels between statin-treated and -untreated patients. Notably, we were only able to detect very low intraplaque RANKL levels, suggesting that this mediator may be preferentially expressed in the blood stream, instead of within atherosclerotic plaques [9]. Our data also suggest a new, potential, beneficial effect of statins as they may directly reduce the levels of circulating free RANKL, thereby preventing its detrimental effects on proatherosclerotic cell subsets (including neutrophils). A potential reduction in both OPG and RANKL serum levels after atorvastatin treatment was previously shown by Dimitrow and coworkers in patients with aortic sclerosis or mild aortic stenosis [22]. In this paper, we confirmed that statin treatment was associated with a reduction in RANKL serum levels, but, surprisingly, we showed an increase in serum OPG (the decoy receptor of soluble RANKL that blocks its bioactivity). The role of OPG in cardiovascular diseases requires further clarifications, since its levels have been associated with cardiovascular risk without reporting a clear proatherosclerotic direct bioactivity of this molecule [23–25]. In $OPG^{-/-}$ mice, treatment with recombinant OPG induced signs of fibrosis, promoted intraplaque SMCs accumulation, collagen fiber formation, and development of fibrous caps, thus supporting a pathogenic role of OPG in the development, progression, and instability of atherosclerotic lesions

Table 6: Spearman rank correlation between serum RANKL/OPG ratio and systemic and intraplaque parameters (upstream or downstream regions) of vulnerability.

	Spearman's correlation coefficient (r)	P-value
Serum RANKL/OPG ratio		
Systemic		
CRP, mg/L	0.3549	0.0311
MPO, ng/mL	0.6036	0.0001
Neutrophil elastase, ng/mL	0.5223	0.0009
MMP-8, ng/mL	0.0436	0.7977
MMP-9, ng/mL	0.3106	0.0614
Pro-MMP-9 activity, ng/mL	0.3248	0.0498
Intraplaque upstream		
versus lipids	−0.3456	0.0489
versus total collagen	0.0077	0.9661
versus collagen I	0.0879	0.6267
versus collagen III	0.0906	0.6162
versus SMCs	−0.0832	0.6452
versus macrophages	−0.0552	0.7605
versus neutrophils	0.1180	0.5132
versus MMP-9	−0.2647	0.1366
Intraplaque downstream		
versus lipids	−0.0946	0.5947
versus total collagen	−0.1383	0.4355
versus collagen I	−0.1636	0.3551
versus collagen III	−0.0805	0.6508
versus SMCs	−0.0118	0.9473
versus macrophages	0.2461	0.1605
versus neutrophils	0.3756	0.0285
versus MMP-9	0.3510	0.0418

[26]. However, additional studies that should be aimed, for instance, at identifying OPG serum cutoff values, are needed to clarify the exact nature of OPG as an innocent bystander or an active player in cardiovascular diseases.

The lack of efficacy of statins in the modulation of intraplaque RANKL/OPG levels might be also explained by different findings. First of all, all statins were administered to the patients from our cohort at a low dose [19]. Thus, their tissue concentration may have been not high enough to reduce intraplaque inflammation. To some extent, this hypothesis is corroborated by the weak improvements in plaque stability parameters that we found in patients on statin as compared to untreated controls. Namely, we only detected a small reduction in neutrophil and MMP-9 content and an increase in intraplaque collagen in the downstream portions of carotid plaques in statin-treated patients versus controls, while Crisby and colleagues [16] had previously found that pravastatin treatment markedly improved carotid plaque stability as compared with control patients by reducing macrophages, T cells, and MMP-2 and by increasing protective intraplaque collagen content.

Another possible reason for our inability to detect a modulation of intraplaque RANKL/OPG levels is that our approach investigated atherosclerotic plaques as very heterogeneous tissues (as a result of different types of shear stress exposure) [20], while Crisby and colleagues evaluated the plaque a single homogeneous tissue. Thus, potential differences from the study may reflect the different approach.

Finally, the fact that different statins (rosuvastatin, simvastatin, and atorvastatin) were administrated to the patients in our study might also explain, at least in part, the limited activity of statins on intraplaque parameters that we observed. A similar result was also reported by Verhoeven and coworkers [27], who found that macrophage content might be particularly affected by atorvastatin treatment but not by other statins.

Notably, in out cohort of patients, treatment with statins did not reduce plaque lipid-rich core size, which is a key vulnerability marker in atherosclerotic plaques. This result may be surprising since Crisby and colleagues [16] clearly demonstrated that the plaques of pravastatin-treated patients with symptomatic carotid artery stenosis had substantially less lipid (3 times less) as compared to control subjects. On the other hand, our findings are supported by a more recent study on coronary atherosclerosis, in which the authors demonstrated that statins significantly decreased plaque size only if LDL-c was <100 mg/mL [28]. In our study, we did not observe such important reduction in LDL-c levels in patients treated with statins (median value 104 mg/dL and LDL-c levels < 100 mg/dL in only 12 patients (46.2%)). Therefore, taking all of these aspects into account and in accordance with the current international recommendations [12], our study suggests that the use of high-dose and long-term lipid-lowering strategy with a statin has to be considered when the goal is to reduce intraplaque vulnerability and cardiovascular adverse events in patients with advanced atherosclerosis [29, 30].

Another important result of this study is represented by the confirmation of serum direct correlations between free RANKL levels and neutrophil products. A significant positive association between levels of RANKL/OPG ratio and other neutrophil products (such as MPO and neutrophil elastase) was also demonstrated, confirming a strong induction of neutrophil degranulation mediated by this cytokine [9]. This robust activation of neutrophils (positively associated in our study with serum RANKL levels) was particularly observed in the systemic circulation instead of within the carotid plaque. Confirming a previous study showing an active role of RANKL in circulating neutrophil degranulation of proatherosclerotic products (such as MMP-9) in patients with increased CAC and *in vitro* [9], this study further confirmed these cells as a major source of proteinases favoring systemic atherosclerotic vulnerability.

This pilot explanatory study has several imitations. Firstly, the number of participants is relatively small (also with a disproportion between statin-treated patients ($n = 26$) and untreated controls ($n = 12$)), thus preventing the detection of small differences in all markers between the two groups. Secondly, as we have mentioned in the discussion, we do not know how different lipid-lowering regimens

(i.e., different statins and high-dose versus low-dose therapeutic approaches) can modulate the inflammatory burden within serum and atherosclerotic plaques. Anyway, our results might unveil a novel "pleiotropic" activity for statins selectively targeting RANKL/OPG system and potentially neutrophil activation and plaque calcification. In conclusion, we demonstrated that patients treated with a statin (low-dose) for severe carotid stenosis had reduced serum levels of free RANKL (RANKL/OPG ratio) as compared to controls without statins. This reduction of RANKL levels was positively associated with a concomitant reduction in neutrophil degranulation products, primarily in the systemic circulation and also within carotid plaques. This hypothesis-generating study may suggest that treatments that more selectively target the RANKL/OPG system might be beneficial in advanced atherosclerosis to reduce neutrophil-mediated patient vulnerability.

Conflict of Interests

All authors declare that no conflict of interests exists.

Acknowledgments

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