Research Article

Comparative Study of Protein Expression Levels of Five Plaque Biomarkers and Relation with Carotid Plaque Type Classification in Patients after Carotid Endarterectomy

Konstantinos Kyriakidis [iD]**,[1,2] Pavlos Antoniadis,[1] Sohail Choksy,[2] and Rigini M. Papi[3]**

[1]*Department of Vascular Surgery, Konstantopoulio General Hospital, "Agia Olga", Athens, Greece*
[2]*Colchester and Ipswich Hospital University Foundation Trust, Colchester, Essex, UK*
[3]*Laboratory of Biochemistry, Department of Chemistry, Aristotle University of Thessaloniki, 54124 Thessaloniki, Greece*

Correspondence should be addressed to Konstantinos Kyriakidis; konstantinos.kyriakidis@colchesterhospital.nhs.uk

Atherosclerosis is an inflammatory process resulting in local plaque deposition in the vessel wall of arteries with symptoms to various areas of vascular tree. Identification of patients with progressive advanced atherosclerotic disease is mainly based on the known characteristics of the vulnerable or recently ruptured plaque. Molecular and cellular features associated with the vulnerable plaque are considered potential diagnostic markers for plaque rupture and thrombosis. Here, protein expression levels of the metalloproteases MMP-1, MMP-9, osteopontin (OPN), and cytokines TNFα and IL-6 in tissue extracts of carotid plaques in patients after endarterectomy were estimated by Western immunoblotting, after SDS-PAGE analysis and evaluated based on the ultrasonographic plaque morphology. The gender and age effect was also examined. MMP-1, MMP-9, and IL-6 were expressed in higher levels compared to OPN and TNFa as well as in symptomatic (with type II and III carotid plaque classification) than asymptomatic (type IV) patients with differences considered statistically significant (P values <0.05). A significant positive correlation between MMP-1 and IL-6 (with Pearson correlation coefficient 0.748) is also notable. The data give further insight into the possible role of specific biomarker and enhance the need for further studies in order to clarify the proper one(s) for detection of the vulnerable plaque and help identify patients at risk for cardiovascular events.

1. Introduction

Atherosclerosis is a chronic, inflammatory disease of the medium and large arteries, such as the aorta and carotid arteries. It is a major contributor to the development of cardiovascular diseases and the leading cause of death worldwide. Although atherosclerotic plaque development is a local process in the vessel wall that can give rise to symptoms in one specific area, it is also a systemic disease with simultaneous plaque formation in different areas of the vasculature [1–3]. Moreover, studies have shown that atherosclerotic carotid arteries pose a substantial risk of ipsilateral cerebrovascular events, with reported annual ischemic stroke rates ranging from 0.35% to 1.3% in asymptomatic patients with moderate stenosis [4, 5] and from 0.5% up to 5% for severe asymptomatic carotid artery stenosis [5, 6].

Atherosclerotic plaques are characterized by intimal thickening from the progressive accumulation of lipids [1, 2] together with other cellular and molecular components such as smooth muscle cells, monocytes, T cells, B lymphocytes, erythrocytes, and platelets. These cells are able to produce and secrete mediator molecules such as cytokines, chemokines, growth-factors, enzymes, and disintegrins, which activate endothelial cells, proliferation of smooth muscle cells, and lesion progression, and contribute to the weakening of a vulnerable plaque by matrix degradation of its fibrous cap. Many of these involved molecules can be measured systemically, and it has been shown that elevated concentrations in the circulation are associated with future cardiovascular events.

In other reports, for example, serum markers such as high-sensitive C-reactive protein, interleukin-6 [7–9], and IL-18 [10] are correlated with the development, progression,

and rupture of atherosclerotic plaque [2, 11]. It is also suggested that inflammatory markers are transcribed locally in atherosclerotic plaques [12–15].

Other markers that have been previously associated with cardiovascular events and are now associated with carotid atherosclerotic plaques are matrix metalloproteinases (MMPs) [16], tissue inhibitor of metalloproteases (TIMP) [17], soluble intercellular adhesion molecule 1 [18], and osteopontin [14, 19]. MMPs are a family of zinc-containing enzymes and are secreted as inactive precursors; they are prevalent in the arterial wall throughout the arterial system and play a central role in degradation of the vascular extracellular matrix resulting in destabilization of the atherosclerotic plaque [16]. The destabilization event contributes to plaque rupture and consequently in acute ischaemic events. Increased carotid MMP-9 plaque levels are associated with an unstable plaque phenotype and are higher in lipid-rich inflammatory plaques. Osteopontin, an acidic phosphoprotein, has recently been demonstrated to inhibit mineral deposition as well as osteoclastogenesis and is constitutively expressed by a wide range of cell types in the vasculature. Carotid plaque contains valuable information for follow-up after vascular surgery. It has been shown that local plaque characteristics are associated with restenosis at the site of carotid endarterectomy after 1 year and endarterectomy of lipid-rich, inflammatory plaques, associated with reduced risk of restenosis compared to stable, fibrous plaques, independent from clinical characteristics [20].

It has been also reported that carotid plaque composition contains predictive information for future cardiovascular events elsewhere in the vascular system, independent from established risk factors and medication. Moreover, patients undergoing carotid endarterectomy, with a local plaque containing intra plaque hemorrhage or marked intra plaque micro vessel formation, demonstrated an increased risk of secondary cardiovascular events with high hazard ratios [21, 22]. Thus, determination of these molecules may give important prognostic information and may in turn be useful in improving risk stratification. However, for most of these biomarkers the clinical utility and their specificity have not yet been established.

This study looks at evaluating carotid plaques in symptomatic and asymptomatic patients after endarterectomy in order to potentially detect vulnerable plaques, based on the differences in protein expression levels of five biomarkers.

2. Material and Methods

2.1. Materials. Rabbit polyclonal antibodies that recognise MMP-1, MMP-9, IL-6, and TNFα were purchased from Acris Antibodies (GmbH, Germany), while anti-osteopontin was purchased from Millipore. The rabbit polyclonal antibody raised against actin and the secondary anti-rabbit antibody conjugated with alkaline phosphatase were purchased from Sigma-Aldrich Chem Co (St. Louis, MO, USA). All other chemicals used in the study were also purchased from Sigma-Aldrich.

2.2. Subjects

2.2.1. Carotid Atherosclerotic Plaque Sample Preparation. 24 patients (14 males, 10 females) age (55-85) were enrolled from the Konstantopoulio General Hospital, Department of Vascular Surgery, Athens, Greece, who had undergone standard carotid ultrasound examination and subsequently carotid endarterectomy. All patients were entered into a prospective database and were on best medical treatment. Colour Doppler ultrasonography and Power Doppler were used in addition to serial ultrasonographic measurements, in order to monitor and evaluate the carotid plaque type and vulnerability preoperatively. Following the carotid endarterectomy, the carotid plaque specimen from each patient was immediately washed and stored at -80°C under liquid nitrogen, until tissue homogenization and subsequent analysis.

The criteria to perform a carotid endarterectomy are based on the recommendations by the ACAS study [23] (Toole JF, 1996) for asymptomatic patients and the NASCET study [24] for symptomatic patients. Sixteen samples (Table 1) were obtained from carotids of symptomatic patients with carotid plaques II and III, and eight samples (Table 1) from asymptomatic patients, all of which had carotid plaque type IV (Table 1). At baseline, clinical parameters, including cardiovascular risk factors and medication used, were recorded. Patients were assigned a unique number in chronological order based on the time of surgical operation of each patient, which is used to identify the samples.

2.2.2. Carotid Ultrasonography. All ultrasound examinations were performed with a Phillips SONOS 5500 equipped with a 7.5MHz linear-array transducer. Initially, the common and internal carotid arteries were scanned in cross-section and longitudinally by B-mode and color Doppler methods, through which the largest plaque was identified for evaluation of plaque echogenicity. Plaque thickness was measured on the longitudinal B-mode or color Doppler images perpendicular to the vascular wall.

2.2.3. Protein Extraction. Frozen carotid plaque samples were homogenized in 50 mM Hepes pH 7.5, 150 mM NaCl, 15 mM β-mercaptoethanol, 0.5 mM phenylmethylsulfonyl fluoride, and 0.1% (v/v) Nonidet P-40 according to Sigala et al. [25]. The homogenates were centrifuged at 2000g for 10 min to obtain total protein extracts that were subsequently assessed by western immunoblotting. Protein concentration in tissue extracts was determined by the Bradford method [26], using bovine serum albumin as the reference standard.

2.2.4. Electrophoresis and Immunoblotting Analysis. Proteins were separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE) using 10% (w/v) polyacrylamide gels containing 0.1% (w/v) SDS as described by Laemmli et al. [27]. Proteins were either stained with Coomassie Brilliant Blue R250 or electrotransferred to nitrocellulose membranes as described by Towbin et al. [28]. Prestained protein markers were used for identification. The immunoblotting analysis was performed using commercially available antibodies raised

TABLE 1: Patient's demographic, clinical characteristics and plaque morphology.

Subject No	Gender	Age	Symptomatic	Asymptomatic	Carotid plaque type
1	M	62	+		II
2	M	69	+		II
3	M	67		+	IV
4	M	78	+		II
5	M	77	+		II
6	F	75	+		III
7	F	74		+	IV
8	F	71	+		III
9	M	58	+		II
10	F	71		+	IV
11	M	79	+		III
12	F	70		+	IV
13	M	61		+	IV
14	F	69		+	IV
15	F	72	+		II
16	M	81	+		III
17	M	83	+		III
18	F	70	+		III
19	F	66		+	IV
20	M	73		+	IV
21	M	77	+		III
22	M	67	+		II
23	M	55	+		II
24	F	78	+		II

against MMP-1, MMP-9, IL-6, TNFα (Acris), and OPN (Millipore). The anti-actin antibody (Sigma-Aldrich) was used for assessing equal loading of total protein per sample. Gel-Pro Analyzer was used to evaluate and record the intensity of each band.

2.2.5. Statistical Analysis. Statistical analysis was performed by SPSS (version 23) software and Microsoft Excel 2011 for Mac and the data were expressed as mean values ± standard deviation (SD). Independent samples T-test was used to determine differences in the variables. P values less than 0.05 were considered significant at confidence level 95%. Pearson correlation analysis was used to assess correlation between the biomarkers.

3. Results and Discussion

Carotid duplex ultrasound provides quantitative measurements of carotid artery stenosis and intima-media thickness. The diagnosis of carotid artery stenosis is important in clinical practice because it determines the need for surgical intervention while carotid intima-media thickness measurements provide a quantitative measure of disease [29]. This study included 24 patients identified as symptomatic (n=16) or asymptomatic (n=8) following a Doppler scan who then went on to have a carotid endarterectomy. Plaque type II was recorded in 9 out of 16 symptomatic patients with prevalence

of men, 7 out of 9. Table 1 lists the patients' demographic and clinical characteristics. The carotid plaques were assessed for the expression levels of the metalloproteases; MMP-1, MMP-9, and OPN and for cytokines TNFa and IL-6. Total proteins of the carotid plaques samples were separated by SDS-PAGE analysis as reported above, followed by Western blotting analysis using MMP-1, MMP-9, IL-6, TNFa, and OPN antibodies. Proteins were analysed on 10% (w/v) SDS-polyacrylamide gels. Prestained protein markers were used for identification. Indicatively, lanes 1-9 (a) and 10-16 (b) of samples 1 to 16 for MMP-1, MMP-9, and IL-6 are shown in Figures 1, 2, and 3, respectively. MMP-9 expression in the tissue extracts gave two bands of lower molecular weight in all samples, symptomatic and asymptomatic, instead of the expected one band at 83 kDa for MMP-9 or at 92 kDa for proMMP-9 showing degradation of MMP-9 in the carotid extracts. On the other hand, IL-6 in the tissue extracts of study samples was detected as a dimer (Figure 3).

Statistical significant differences in MMP-1 levels between symptomatic and asymptomatic patients, P<0.05 (P=0), were recorded (Figure 4(a)).

Higher protein levels of MMP-9 were also detected in the samples of symptomatic than in asymptomatic patients (Figure 4(b)), with the differences being statistically significant P<0.05. Our data are in agreement with previous studies showing that MMPs, including MMP-1, MMP-3, and MMP-9, were overexpressed in human atherosclerotic plaques and
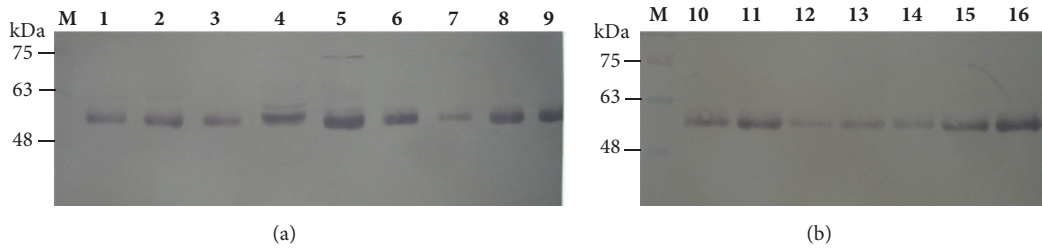
FIGURE 1: Western blot analysis of proteins using MMP-1 antibody. Proteins were analysed on 10% w/v SDS-polyacrylamide gels. M: prestained protein markers, lanes 1 -9 (a), 10-16 (b): 50 μg total proteins.
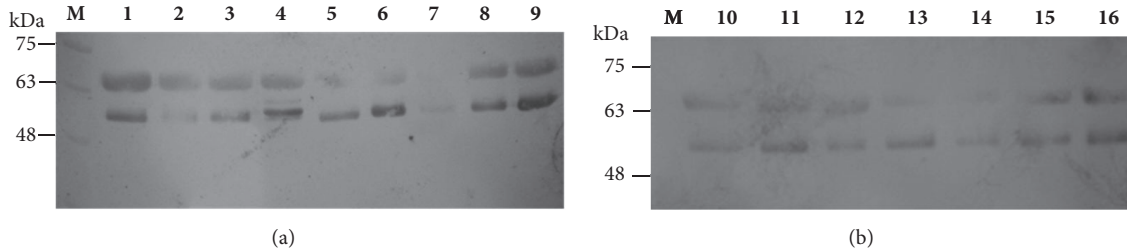

FIGURE 2: Western blot analysis of proteins using MMP-9 antibody. Proteins were analysed on 10% w/v SDS-polyacrylamide gels. M: prestained protein markers, lanes 1-9 (a) and 10-16 (b): 50 μg total proteins.
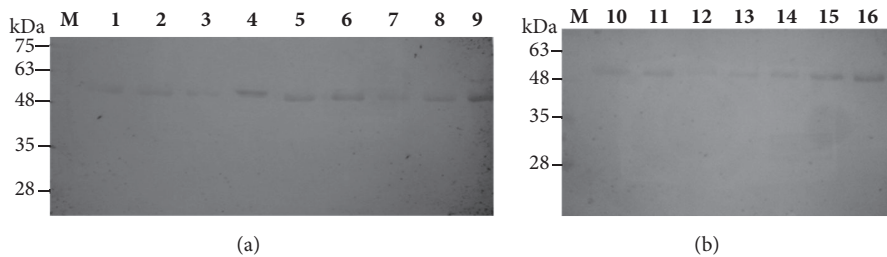

FIGURE 3: Western blot analysis of protein expression using IL-6 antibody. Proteins were analysed on 10% w/v SDS-polyacrylamide gels. M: prestained protein markers, lanes 1-9 (a) and 10 -16 (b): 50 μg total proteins.

in animal models and this is particularly associated with macrophages [30]. Elevated levels of IL-6 were observed more prominently in symptomatic (with plaque type II/III) versus asymptomatic patients (plaque type IV) as can be seen in Figure 4(c); the observed differences are statistically significant (P=0). Furthermore, studies of functional genetic polymorphisms in genes encoding five MMPs (MMP-1, MMP-3, MMP-7, MMP-9, and MMP-12) in relation to subclinical atherosclerosis phenotypes revealed a strong association of MMP-9 279Q allele and the presence of plaques in men and specifically carotid plaques [31]. A gender-specific effect of another MMP-9 functional polymorphism and carotid arterial stiffness was also reported by Lin et al. [32]. Here, no significant gender effect is found, in symptomatic and asymptomatic samples. However, a larger population with sample variety in addition to simultaneously studying the impact of genetic variation and protein expression level of MMP-9 is a study we plan to conduct.

The expression level of OPN was low and the variation within samples from symptomatic and asymptomatic patients was statistically insignificant at a confidence level 95%, with P=0.2. Previous reports show that carotid plaque osteopontin level is a predictor of cardiovascular events after carotid endarterectomy [33] and high levels of it recorded in serum [34]. On the other hand, lower OPN levels result in mineral deposition and plaque calcification and, therefore, enhance plaque stability and decrease the likelihood of clinical events [35].

Cytokines are key regulatory glycoproteins that modulate all aspects of vascular inflammation. Many cytokines have been implicated in atheroma formation and complication. In this study, the cytokines IL-6 and TNFa were measured. IL-6 is a phosphoglycoprotein with MW ranging from 21 to 29 kDa, depending on the degree of glycosylation and phosphorylation and IL-6 dimer forms a complex with the dimer of IL-6 receptor and the dimer of gp-130 to initiate signal transduction [36, 37]. IL-6 enhances cell adhesion molecule expression as well as the production of acute phase reactants such as CRP and TNFa by the hepatocytes. Higher IL-6 levels are associated with lower echogenicity of carotid plaques suggesting a link between inflammation and potential risk of plaques [38]. Epidemiological studies have
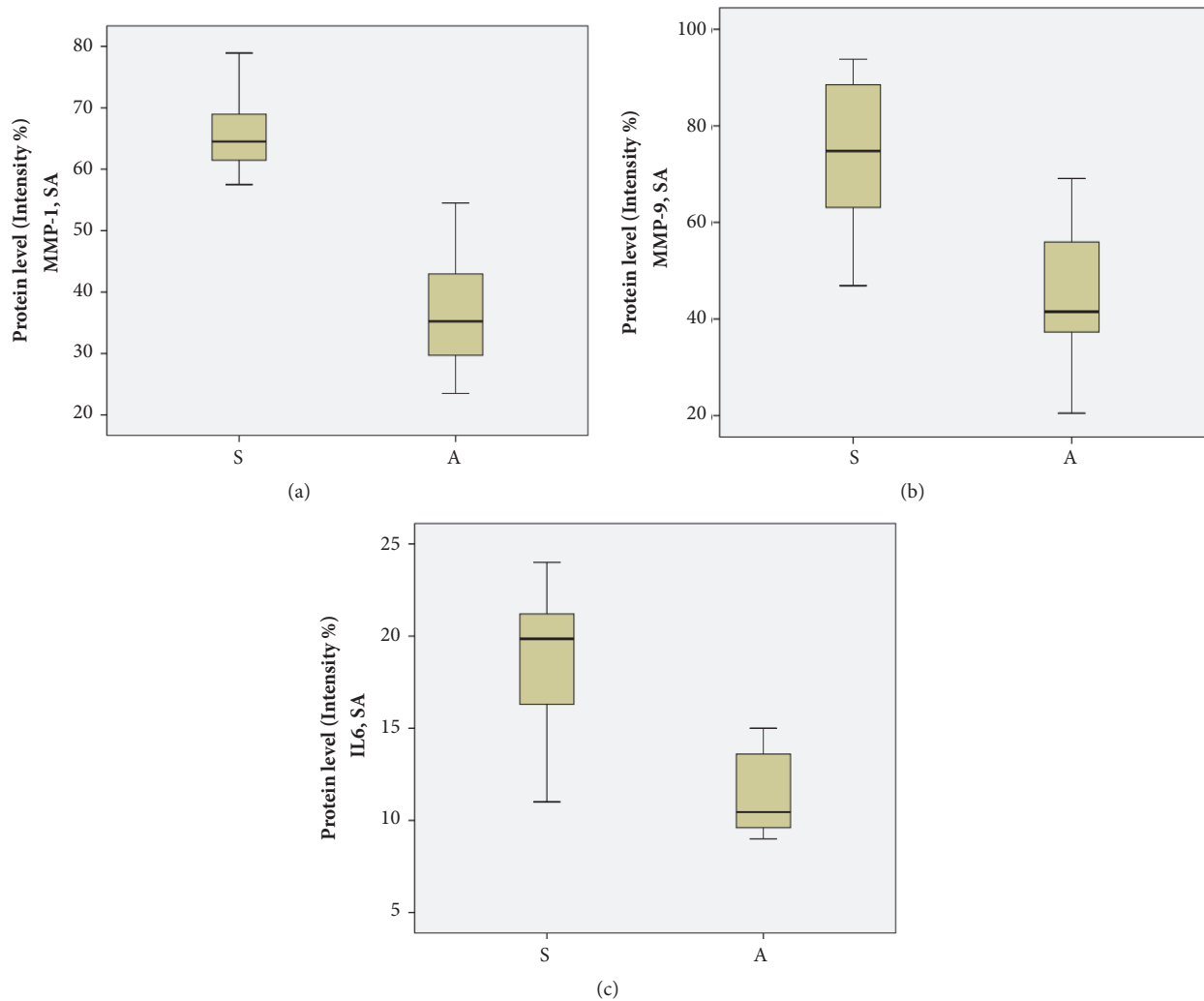
Figure 4: Boxplot depiction of differences in MMP-1 (a), (MMP-9) (b) and IL-6 (c) values between symptomatic and asymptomatic patients (P=0).

found increased vascular risk in association with increased basal levels of cytokines IL-6 and TNFa, cell adhesion molecules such as soluble ICAM-1, P selectin, E selectin, and downstream acute-phase reactants such as CRP, fibrinogen, and serum amyloid A [39]. The proinflammatory protein levels, TNFa, are also related to carotid atherosclerosis [40]. Here, TNFa was detected as a dimer, as in the case of IL-6, though expressed at low yields in all samples and no statistically significant differences in the mean values between symptomatic and asymptomatic samples were observed, with P=0.09 at a confidence level 95% (data not shown).

Expression levels of these five biomarkers, both in symptomatic and asymptomatic patients, were also evaluated regarding the gender of patients. There is no statistical significant difference between males (n=11) and females (n=5) in the values of protein expression levels in all cases of symptomatic patients. The boxplot of the mean MMP-1 values in male and female symptomatic patients, indicatively, is shown in Figure 5. Gender differences in asymptomatic patients with atherosclerotic carotid plaques are reported by

Ota et al. [41]. Furthermore, no significant differences were recorded in the expression levels of the biomarkers versus the age of patients both symptomatic and asymptomatic ones. The effect of age (in the range of 55-85 years) on MMP-1 values, indicatively, of male (MMP-1M) and female (MMP-1F) symptomatic patients is shown in the scatterplot of Figure 6. Similar studies were also conducted with the other four biomarkers (data not shown). It can be said that in patients over 70 years of age, the values of the expression levels of all biomarkers and specifically of MMP-1, MMP-9, and IL-6 are elevated and the differences between male and female values tend to coincide. This is in agreement with previous report indicating that there is no age effect [42].

Pearson correlation values were used to examine linear correlation between the biomarkers. Pearson correlation coefficient value of 0.748 for MMP-1 and IL-6 and 0.606 for MMP-9 and IL-6 (Table 2) indicates high levels of correlation between them, while that of 0.594 for MMP-1 and MMP-9 indicates a moderate level. Values are significant in the 0.01 level (two-tailed). The differences in mean values of
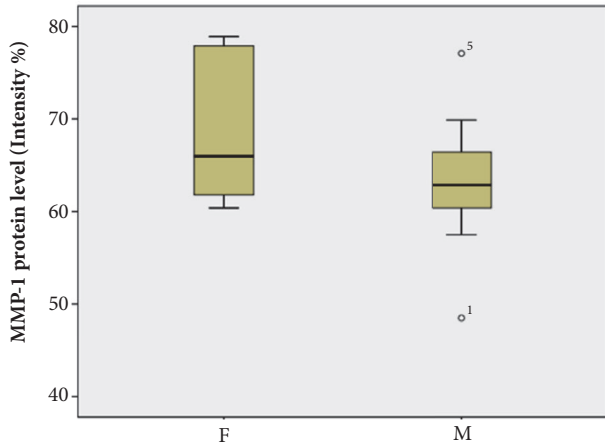
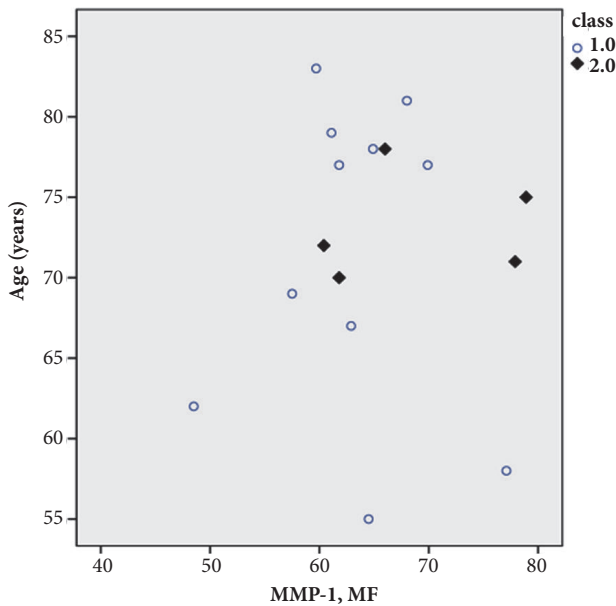FIGURE 5: Boxplot depiction of MMP-1 values of male and female symptomatic patients (P>.05).

FIGURE 6: Scatterplot analysis of MMP-1 levels in male (O) and female (♦) versus age in symptomatic patients.

TABLE 2: Pearson correlation coefficient values between the biomarkers.

	MM-1	MMP-9	TNFa	IL-6	Osteopontin
MMP-1	1				
MMP-9	0.594[a]	1			
TNFa	0.164	0.399	1		
IL-6	0.748[a]	0.606[a]	0.172	1	
Osteopontin	0.365	0.476[b]	0.469[b]	0.116	1

[a]Correlation is significant at the 0.01 level (2-tailed)
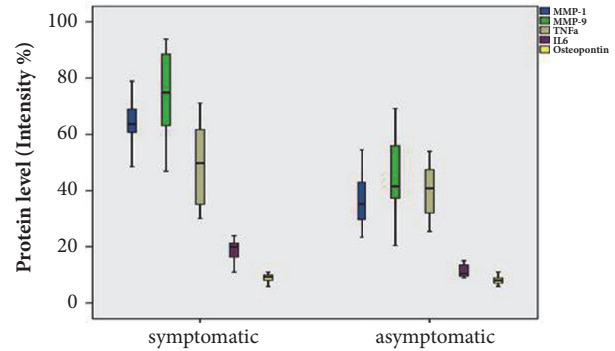[b]Correlation is significant at the 0.05 level (2-tailed)

FIGURE 7: Boxplot of the mean values of the protein levels of all the biomarkers for symptomatic and asymptomatic patients.

findings support that elevated levels of the three biomarkers identify symptomatic patients and may be used for vulnerable plaque classification and the prediction of patients' risk for cardiovascular events.

It is worth mentioning that despite the prominent differences in the protein expression level of the biomarkers studied, no control samples from healthy individuals could be used in order to have a threshold value to compare with, indicating that a standard control when evaluating a biomarker is still of major concern. This is due to obvious ethical issues and carotid plaque analysis is therefore limited to patients who undergo vascular surgery.

Thus, these data analyses prompt further studies, evaluating larger groups of patients and compare the protein expression levels of the biomarkers in carotid plaques versus the circulating ones which may give rise to tissue specific biomarkers. Therefore, it may provide evidence towards patient selection and the treatment planning in order to decrease the perioperative risk.

Data Availability

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

Disclosure

The present address for Konstantinos Kyriakidis and Sohail Choksy is Colchester and Ipswich Hospital University Foundation Trust, Colchester, Essex, UK.

protein expression levels of the five biomarkers between symptomatic and asymptomatic patients are shown in the box plot in Figure 7. Further attempt to correlate the levels of the biomarkers in symptomatic patients, with the specific plaque types II and III, did not show any clear association, if there is such (data not shown), due to the moderate number of samples included in each case.

4. Conclusion

The results demonstrate that expression levels of MMP-1, MMP-9, and IL-6 were significantly more pronounced than OPN and TNFa, respectively, and statistical significant differences in the values of the first three between symptomatic and asymptomatic patients were also observed. The

Conflicts of Interest

Acknowledgments

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