Original Paper

Cerebrovascular Diseases

Cerebrovasc Dis 2009;28:601–610
DOI: 10.1159/000247605

Received: April 21, 2009
Accepted: July 21, 2009
Published online: October 16, 2009

Multiple Biological Predictors for Vulnerable Carotid Lesions

Jaroslav Pelisek[a] Martina Rudelius[b] Peter Zepper[c] Holger Poppert[c]
Christian Reeps[a] Tibor Schuster[d] Hans-Henning Eckstein[a]

Departments of [a]Vascular Surgery, [b]Pathology and [c]Neurology, and [d]Institute of Epidemiology and Medical Statistics (IMSE), Klinikum rechts der Isar der Technischen Universität München, Munich, Germany

Key Words
Carotid artery · Vulnerable plaques · Biomarker · Matrix metalloproteinases · Inflammatory factors

Abstract
Background: In this study a multiscore analysis of various biomarkers including matrix metalloproteinases (MMPs), inflammatory factors and other clinical parameters was performed to establish a set of reliable biomarkers for improved detection of plaque instability in patients with advanced carotid stenosis. ***Methods:*** Study patients (n = 101) were classified as histologically stable (n = 37) or unstable (n = 64). Serum levels of MMP-1, -2, -3, -7, -8, -9, MMP inhibitors TIMP-1, -2, and inflammatory factors such as tumor necrosis factor (TNF-α), interleukin (IL)-1β, -6, -8, -10, and -12 were measured by ELISA assays. Multiscore analysis was performed using multiple receiver operating characteristics analysis and determination of appropriate cutoff values. ***Results:*** Circulating levels of MMP-1, -7, TIMP-1, TNF-α, and IL-8 were significantly enhanced in patients with unstable plaques compared to individuals with stable lesions, mean differences being 1.2 (p = 0.032), 2.5 (p = 0.004), 30.0 (p = 0.014), 1.3 (p = 0.047), and 2.2 (p = 0.033), respectively. The combination of MMP-1, -7, TIMP-1 and IL-8 demonstrated the highest positive predictive value of 89.4% and negative predictive value of 60.1% for patients correctly classified as individuals with unstable and stable carotid lesions by means of blood sample analysis. ***Conclusions:*** Multiple relevant biomarkers that play a decisive role in plaque instability can improve the correct determination of vulnerable carotid plaques in patients with advanced carotid artery stenosis.

Introduction

Advanced atherosclerotic lesions of carotid arteries are highly vulnerable and often lead to ischemic events [1–4]. Degradation of extracellular matrix components particularly by matrix metalloproteinases (MMPs), together with an increased inflammatory reaction is the main reason for plaque progression to rupture and consequent neurological symptoms [3, 5, 6]. Thus, MMPs and inflammatory factors may also serve as possible markers of plaque instability [7–12]. Several studies have already demonstrated increased levels of some MMPs and inflammatory proteins in the blood of patients with advanced carotid atherosclerosis [13–18]. However, most studies investigated these biomarkers in patients with symptomatic or asymptomatic carotid stenosis and in patients with or without emboli [13, 16–18]. Only few stud-

KARGER

Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

1015–9770/09/0286–0601$26.00/0

Accessible online at:
www.karger.com/ced

Dr. Jaroslav Pelisek, PhD
Department of Vascular Surgery
Klinikum rechts der Isar der Technischen Universität München
Ismaninger Strasse 22, DE–81675 Munich (Germany)
Tel. +49 89 4140 5168, Fax +49 89 4140 4861, E-Mail pelisek@gmx.de

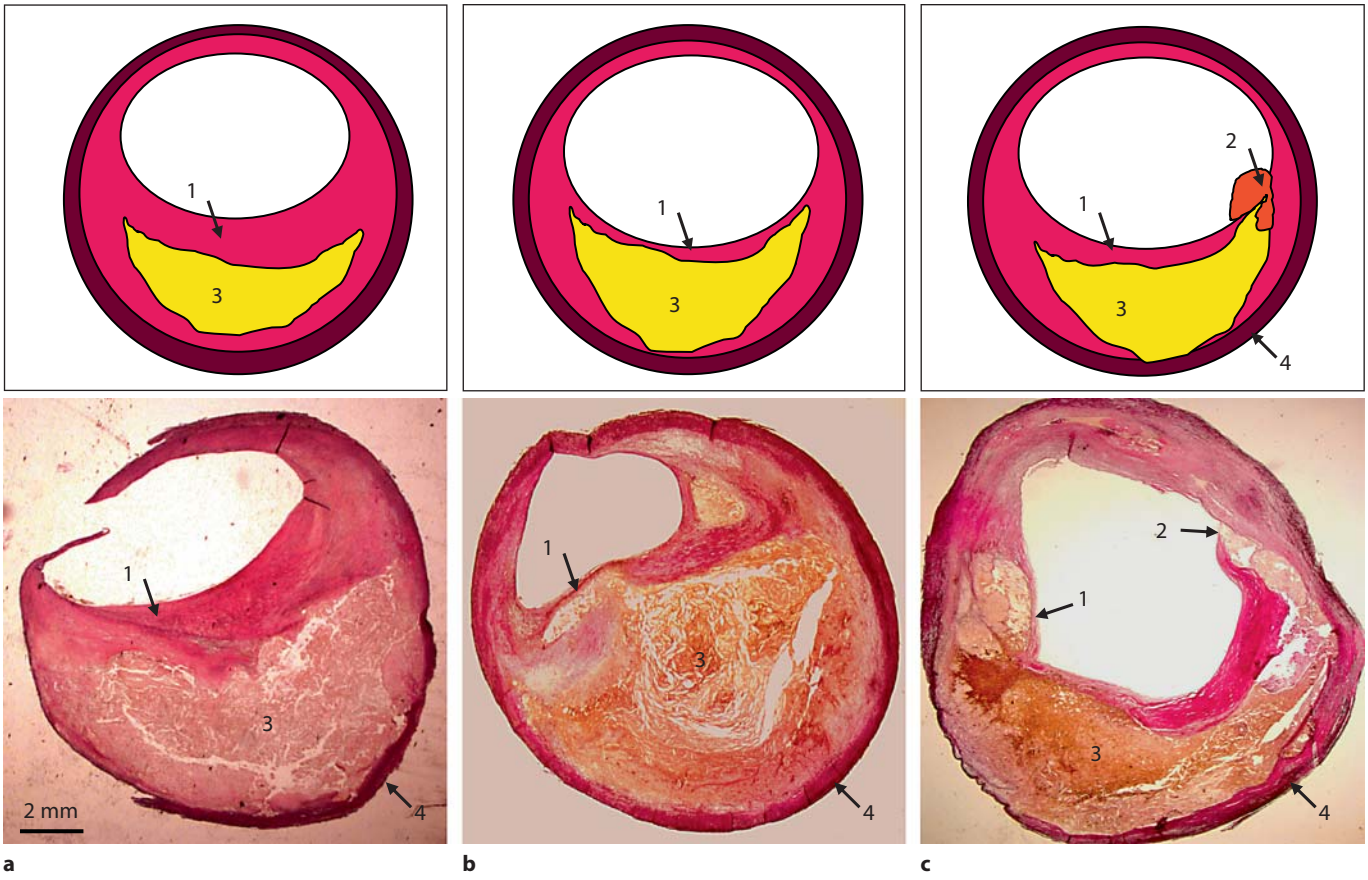

Fig. 1. Schematic drawings and selected examples of advanced atherosclerotic lesions in the intimal region of patients with advanced carotid artery stenosis following CEA: stable (**a**), unstable (**b**), and ruptured carotid plaques (**c**). 1 = Fibrous cap, 2 = rupture site, 3 = necrotic atheromatous core, 4 = remaining medial part after CEA.

ies have analyzed e.g. MMPs in the blood of patients with stable or unstable plaques determined by morphological criteria [13, 15]. Furthermore, only very few investigations have evaluated the usefulness of multiple biomarkers to predict rupture-prone lesions [19–21].

The aim of the study was therefore to analyze various soluble MMPs (MMP-1, -2, -3, -7, -8, -9), tissue inhibitor metalloproteinases (TIMP-1, -2), inflammatory factors such as C-reactive protein [CRP, fibrinogen, tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, -6, -8, -10, -12] and other clinical parameters (hypertension, diabetes mellitus, hypercholesterolemia, nicotine abuse, renal failure, coronary heart disease, urea nitrogen, creatinine, creatine kinase, leukocytes, thrombocytes and erythrocytes) to find an optimal set of biomarkers of vulnerable plaques in patients with advanced carotid stenosis for possible prediction of ischemic stroke.

Patients and Methods

Patients

The study group consisted of 145 consecutive patients with high-grade carotid artery stenosis intended for carotid endarterectomy (CEA). Carotid plaques of these patients were analyzed by means of histology and characterized as stable or unstable (see further below). Patients with plaques that could not be clearly defined as stable or unstable or ruptured were excluded from the study. Thus, 101 patients with histologically well-defined carotid plaque morphology were included: n = 37 patients with stable carotid lesions and n = 64 patients with unstable or ruptured plaques. The degree of stenosis was evaluated by color-coded duplex sonography following ECST criteria in all patients. All patients underwent detailed neurological examination within 2 days before and after the procedure by a neurologist. Fifty-seven patients had experienced a previous neurologic event, the remainder had no symptoms. The study was performed according to the Guidelines of the World Medical Association Declaration of Helsinki.

Table 1. Demographic and clinical characteristics of study patients (%)

	Stable (n = 37)	Unstable (n = 64)	p value
Mean age ± SD, years	68.9 ± 9.6	68.8 ± 8.6	0.957
Sex, male/female	56.8/43.2	71.9/28.1	0.136
Neurological symptoms	32.4	68.8	0.045*
Associated diseases			
Hypertension	93.8	81.1	0.103
Diabetes mellitus	18.8	27.6	0.353
Hypercholesterolemia	87.5	73.8	0.278
Nicotine abuse	40.6	53.5	0.247
Renal failure	6.3	6.9	0.908
Coronary heart disease	32.3	37.9	0.597
Medication			
Aspirin/clopidogrel	99.7	98.1	0.457
Beta-blockers	64.5	71.4	0.507
ACE inhibitors	38.7	48.2	0.314
Statins	58.1	58.9	0.938
Nitrates	9.7	7.4	0.449

Carotid Artery Classification and Determination of Plaque Stability

The carotid plaque was removed by standard CEA, fixed in formalin, separated into 3–5 segments of 3–4 mm (depending on plaque size) and embedded in paraffin. From each segment sections of 2–3 μm were prepared and routinely stained with hematoxylin and eosin and elastin van Gieson to assess plaque structure, stability, and fibrous cap thickness. Stained samples were analyzed using light microscopy by 2 independent experts (one of them was an experienced pathologist) blinded as to the study groups. Morphological characteristics of the carotid plaques were established according to the classification of the American Heart Association. All plaques were advanced lesions graded as type V, VI, VII, or VIII [22]. Selection of stable or unstable plaques was performed in accordance with the modified scheme established by Virmani et al. [3, 23]. Carotid lesions were classified by 2 independent and experienced investigators as follows: unstable lesions = ruptured or rupture-prone plaques with thin-cap fibroatheroma of cap thickness less than 65 μm; stable lesions = plaques with thick-cap fibroatheroma and/or without lipid/necrotic core. A schematic drawing and illustrative figures of stable and unstable or ruptured carotid plaques are given in figure 1.

Enzyme-Linked Immunosorbent Assay and Sample Analyses

MMPs and TIMPs were quantified in serum samples using enzyme-linked immunosorbent assays (ELISA) from R&D System (Quantikine human MMP-1, -2, -3, -7, -8, -9 and TIMP-1, -2 kit; Wiesbaden-Nordenstadt, Germany) according to the manufacturer's protocol. The color development was measured by using multiplate reader Mithras LB940 (Berthold Technologies, Bad Herrenalb, Germany) at 450 nm with correction at 570 nm. Inflammatory markers were analyzed using Cytometric Bead Array (BD Biosciences, San Jose, Calif., USA). The BA system uses the sensitivity of amplified fluorescence detection by flow cytometry in a particle-based immunoassay. Special beads provide a capture surface with distinct fluorescence intensities specific for TNF-α, IL-1β, -6, -8, -10, -12 and are analogous to the individually coated wells in the ELISA plate. Fibrinogen activity was determined by the Clauss method (Dade Behring, Schwalbach, Germany). High-sensitivity CRP was determined by ELISA assay from Life Diagnostics (West Chester, Pa., USA) with an analytical sensitivity of <0.01 mg/dl. Determination of other clinical markers such as urea nitrogen, creatinine, creatine kinase, leukocytes, thrombocytes, erythrocytes and other blood sample parameters was performed using routine assays in laboratories of our clinical chemistry.

Immunohistochemistry

Histological analyses and immunohistochemistry were performed on formalin-fixed paraffin-embedded sections of carotid plaques. The sections were routinely stained with hematoxylin and eosin and elastin van Gieson to assess the plaque structure, stability, size of the necrotic core, fibrous cap thickness, cellular composition of the plaques and degree of infiltration. For immunohistochemistry anti-CD68 (macrophages/monocytes; clone KP1, dilution 1:2,000; Dako) and anti-CD45 (infiltrates/lymphocytes; mouse monoclonal antibody, dilution 1:30; Novocastra, UK) primary antibodies were used. Following primary antibody incubation, visualization was performed by using the Peroxidase/DAB ChemMate Detection Kit (biotinylated goat anti-mouse/anti-rabbit secondary Ab; Dako) according to the manufacturer's instructions.

Statistical Analysis

All statistical analyses were performed using SPSS for Windows version 15.0 (SPSS Inc., Chicago, Ill., USA). Values of continuous variables were expressed as means ± standard deviation (SD). Kolmogorov-Smirnov test was used to assess the deviation from normal distribution of continuous data. In case of non-normal skewed distribution of data log transformation was conducted if appropriate. To compare means between two independent normally distributed samples, t test was applied. For all other two-sample comparisons the nonparametric Mann-Whitney U test was used. Correction for multiple testing was performed by Benjamini and Hochberg [24] approach that considers the fraction of false positives over the amount of tests declared significant and does not reduce the overall statistical power. Correlations between continuous variables were quantified by using Spearman's rank correlation coefficient. Receiver operating characteristic (ROC) analysis was applied to evaluate and compare the performance of group prediction (stable or unstable carotid lesion) by biomarkers. The resulting sensitivity and specificity were used to determine optimal cutoff values of each prognostic marker and their combinations by using Youden index [J = maximum (sensitivity + specificity – 1)]. Positive and negative predictive values were used to assess the accuracy of prediction. All statistical comparisons were performed two-sided in the sense of an exploratory data analysis using a 0.05 (*), 0.01 (**), and 0.001 (***) level of significance.

Table 2. Serum levels of MMP, TIMPs, inflammatory and other clinical factors

	Stable (n = 37)	Unstable (n = 64)	p value[1]	Corrected p value[2]
MMP-1	2.9 ± 2.5 (0.3–10.5)	4.1 ± 3.3 (0.39–17.8)	0.032*	0.100
MMP-2	252 ± 76 (159–471)	268 ± 88 (139–542)	0.437	0.680
MMP-3	13.4 ± 4.9 (7.3–25.9)	13.9 ± 8.1 (7.1–48.8)	0.876	0.876
MMP-7	8.3 ± 4.4 (0.4–22.6)	10.8 ± 4.3 (4.9–28.4)	0.004*	0.016**
MMP-8	10.4 ± 8.3 (1.4–32.4)	14.8 ± 16.4 (1.9–67.1)	0.202	0.354
MMP-9	191 ± 61 (74–325)	203 ± 79 (78–411)	0.437	0.644
TIMP-1	147 ± 40 (70–229)	177 ± 66 (82–389)	0.014*	0.049**
TIMP-2	60.4 ± 23.4 (25.7–109.6)	74.9 ± 37.1 (7.8–161.1)	0.058	0.135
TNF-α	3.6 ± 0.9 (1.7–5.3)	4.9 ± 3.15 (0–14.9)	0.047*	0.120
IL-1β	1.4 ± 2.0 (0–7.2)	2.2 ± 3.8 (0–15.4)	0.444	0.622
IL-6	2.9 ± 0.9 (1.5–4.7)	3.7 ± 2.7 (1.5–10.1)	0.193	0.386
IL-8	5.9 ± 4.2 (3.5–20.9)	8.1 ± 5.4 (2.6–22.9)	0.033*	0.092
IL-10	1.5 ± 1.0 (0–3.1)	2.5 ± 2.2 (0–9.7)	0.058	0.125
IL-12	1.4 ± 2.1 (0–7.8)	2.1 ± 4.5 (0–17.7)	0.530	0.707
hsCRP	1.4 ± 2.1 (0.1–8.0)	1.1 ± 1.0 (0.01–5.3)	0.549	0.699
Fibrinogen	353 ± 84 (242–594)	372 ± 107 (217–731)	0.434	0.715
Urea	17.9 ± 5.4 (9.0–29.0)	18.7 ± 8.9 (4.6–53.0)	0.759	0.886
Creatinine	0.9 ± 0.3 (0.6–2.3)	1.1 ± 0.4 (0.7–2.8)	0.199	0.371
Creatine kinase	95 ± 51 (37–230)	98.6 ± 50.3 (32–280)	0.812	0.842
Leukocytes	7.4 ± 1.7 (4.1–12.7)	7.2 ± 1.8 (4.0–12.2)	0.561	0.683
Erythrocytes	4.6 ± 0.5 (3.1–5.4)	4.5 ± 0.5 (3.2–5.4)	0.810	0.872
Thrombocytes	235 ± 41 (142–324)	245 ± 74 (115–504)	0.778	0.871

Data are shown as mean ± SD with range in parentheses. MMPs and TIMPs: ng/ml; TNF-α, IL-1β, IL-6, -8, -10, -12: pg/ml; high-sensitivity CRP (hsCRP), fibrinogen, urea, and creatinine: mg/dl; leukocytes, thrombocytes: $10^3/\mu l$; erythrocytes: $10^6/\mu l$.

[1] Significant differences between groups by using single comparison (*$p < 0.05$).

[2] Multiple correction analysis by using Benjamini and Hochberg approach (**$p < 0.01$).

Results

Patient Demographic Data

The characteristics of patients, various risk factors, associated diseases and other clinical symptoms are summarized in table 1. Both groups, patients with stable and unstable carotid lesions, had no significant differences with regard to patient epidemiology, associated diseases or medication. The only significant difference was found for neurological symptoms. Patients with unstable plaques had 2.1 times more frequently neurological symptoms compared to individuals with stable lesions ($p = 0.045$). The average age of the study population was 68.9 ± 9.1 years. A predominant number of patients were hypertonic (87.4%) and hypercholesterolemic (80.6%). Almost all patients received aspirin (300 mg) or clopidogrel (75 mg) (98.9%).

Determination of Serum Levels of MMPs, TIMPs, Inflammatory Factors and Other Clinical Parameters

The concentrations of MMPs and TIMPs in the serum of patients with stable and unstable carotid plaques are summarized in table 2. MMP-2, -3, -8, -9 and TIMP-2 were not significantly different between the groups. In contrast, the levels of circulating MMP-1, -7, and TIMP-1 were significantly enhanced in individuals with unstable lesions [mean differences in ng/ml: 1.2 ($p = 0.032$), 2.5 ($p = 0.004$), and 30.0 ($p = 0.014$), respectively; fig. 2]. Regarding inflammatory factors only TNF-α and IL-8 showed a significant increment of 1.3 and 2.2 pg/ml in patients with unstable carotid lesions ($p = 0.047$ and 0.033, respectively; table 2, fig. 2) compared to patients with stable carotid lesions. The remaining inflammatory markers and other clinical factors such as CRP, fibrinogen, urea, creatinine, creatine kinase, leukocytes, eryth-

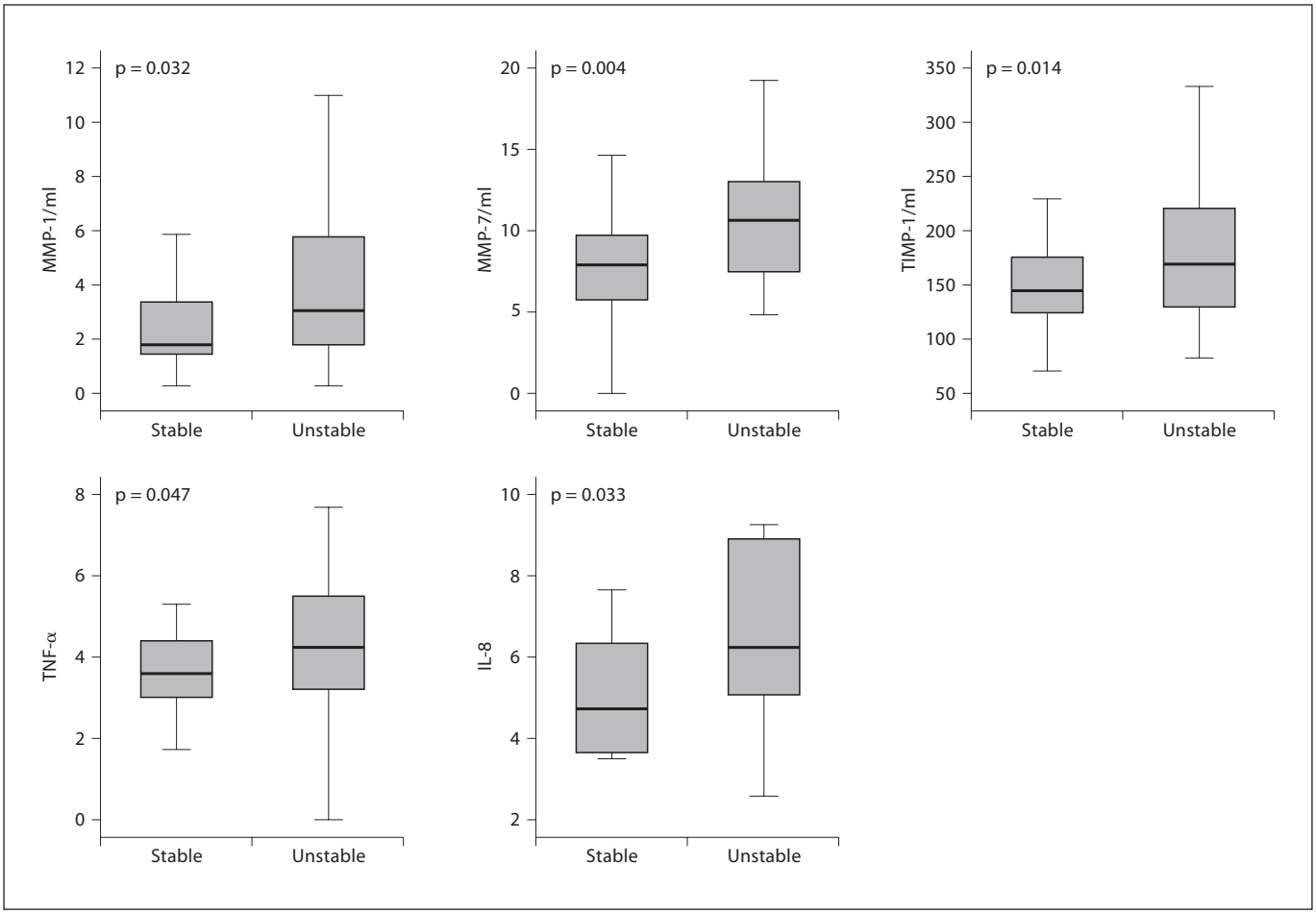

Fig. 2. Comparison of MMP-1, -7, TIMP-1, TNF-α, and IL-8 values in blood serum of patients with stable versus unstable carotid lesions using box-and-whisker plots with significant differences between the groups (table 2). The boxes demonstrate values between 0.25 and 0.75 quartiles with the median within the box. The lines (whiskers) show 95% percentiles of the corresponding samples.

rocytes and thrombocytes revealed no differences between the groups. Furthermore, to adjust the significance values for multiple biomarkers, we performed an appropriate correction for multiple testing by using Benjamini and Hochberg approach (table 2). Following correction, MMP-7 and TIMP-1 were still statistically significant between the groups ($p = 0.016$ and $p = 0.049$, respectively).

Multiple ROC Curve Analysis and Cutoff Values

First, a ROC curve was designed for all biomarkers considered in the study. In accordance with the above-described analyses of differences between patients with stable and unstable carotid lesions, only MMP-1, -7, TIMP-1, TNF-α, and IL-8 showed curves above the non-discrimination line. The sensitivity, specificity and the predictive values are summarized in table 3. From the ROC curve the cutoff value for each biomarker was determined by using Youden index (giving the cutoff value with the highest accuracy of prediction) to distinguish between patients with stable and unstable carotid lesions. The cutoff values were as follows: MMP-1 = 1.88 ng/ml; MMP-7 = 10.66 ng/ml; TIMP-1 = 196.2 ng/ml; TNF-α = 4.70 pg/ml; IL-8 = 4.8 pg/ml. All patients with biomarker values higher than the cutoff points were considered as individuals with unstable plaques. Furthermore, multiple ROC analysis was performed by combination of the above-described biomarkers. The significance values between patients with stable or unstable carotid plaques markedly increased when the corresponding biomarkers were combined (table 3). Indepen-

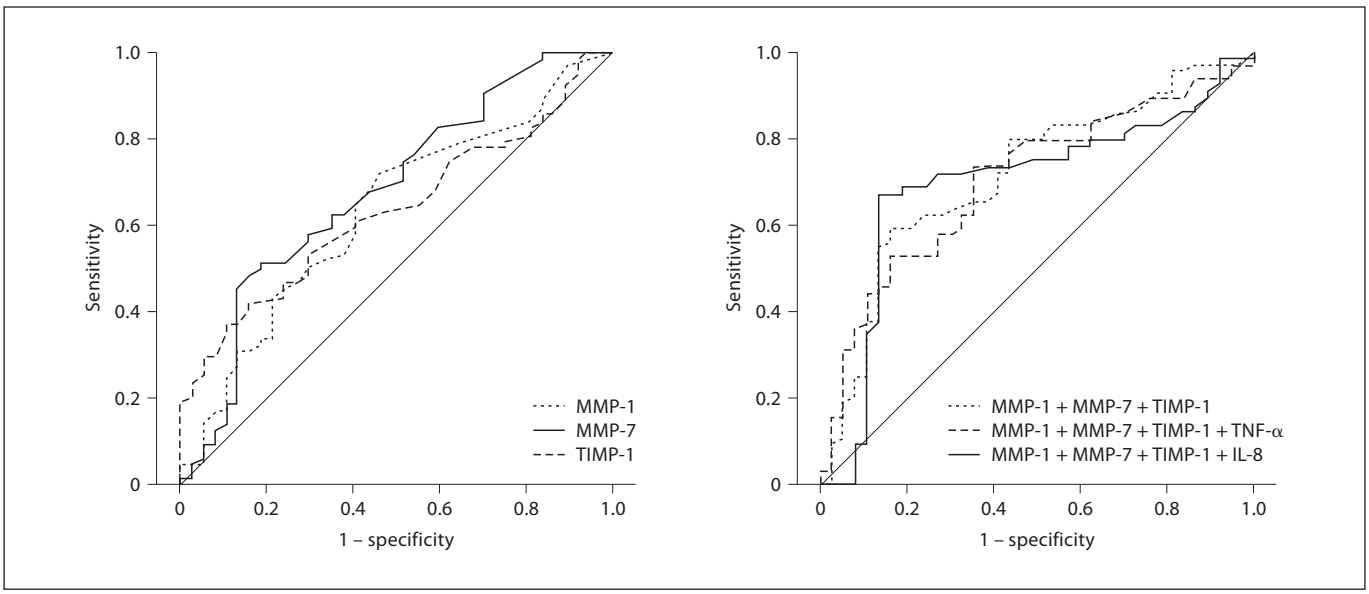

Fig. 3. ROC curves. Comparison of the performance of biomarkers MMP-1, MMP-7, and TIMP-1 alone, each significantly different between patients with stable and unstable carotid lesions, and the combination of MMP-1 + MMP-7 + TIMP-1 with and without TNF-α or IL-8. The curve above the diagonal shows the true-positive against the false-negative rate for determination of possible cutoff values of a prediction algorithm as a trade-off between sensitivity and specificity. The best performance and the highest predictive values were found for the combination of MMP-1 + MMP-7 + TIMP-1 + IL-8 with a PPV of almost 90% and an NPV of 60% (table 3).

dent of the multiple testing corrections of these multiscore biomarkers, the p values were still highly significant. The best performance and the highest predictive values were found for the combination of MMP-1 + MMP-7 + TIMP-1 + IL-8 with positive predictive value (PPV) of almost 90% and negative predictive value (NPV) of 60% (table 3). Figure 3 shows the ROC curves for individual biomarkers and their combinations. Multiple biomarkers showed markedly improved performance of the ROC curve. Furthermore, the distance between the nondiscrimination line and the top of the curve was significantly greater by the combination of biomarkers as compared to single biomarkers.

Semiquantitative Evaluation of Infiltrates and Macrophages in Carotid Plaques

For evaluation of possible correlations between the level of MMPs and inflammatory factors in the blood of patients with advanced carotid stenosis and the degree of inflammation within the plaques, semiquantitative analysis of inflammatory cells and macrophages was performed. Significant correlations were observed between the number of macrophages and levels of IL-1β or IL-8 in the blood ($r = 0.376$, $p = 0.049$ or $r = 0.505$, $p = 0.006$). In contrast, no correlation was found between the amount of inflammatory cells determined by CD45 and MMPs or inflammatory factors.

Correlation between MMPs, TIMPs, Inflammatory Factors and Other Clinical Parameters

Finally, correlation study was performed between MMPs, TIMPs and all other factors in the blood of patients with advanced carotid artery stenosis by using Spearman's rank correlation coefficient (table 4). Various correlations were found between individual MMPs, MMPs and TIMPs, MMPs and inflammatory factors, and MMPs and fibrinogen, leukocytes, erythrocytes, and thrombocytes. The highest correlations were observed between MMP-8 and MMP-9 ($r = 0.695$), MMP-2 and TIMP-2 ($r = 0.545$), TIMP-1 and TIMP-2 ($r = 0.443$), fibrinogen and MMP-8 and MMP-9 ($r = 0.441$ and 0.442, respectively), and MMP-9 and leukocytes ($r = 0.343$). Interestingly, various MMPs (MMP-7, -8, -9, and TIMP-1) correlated also with fibrinogen (table 4). Furthermore, significant correlations were observed also between individual inflammatory factors, e.g. IL-8 and IL-12 correlated with all other inflammatory factors tested.

Table 3. Correlation between MMPs, TIMPs and other clinical parameters

	MMP-1	MMP-2	MMP-3	MMP-7	MMP-8	MMP-9	TIMP-1	TIMP-2
MMP-1	–							
MMP-2	w.c.	–						
MMP-3	w.c.	w.c.	–					
MMP-7	0.302**	w.c.	0.325**	–				
MMP-8	w.c.	w.c.	w.c.	w.c.	–			
MMP-9	w.c.	w.c.	w.c.	w.c.	0.729***	–		
TIMP-1	0.222*	0.197*	w.c.	0.366**	w.c.	w.c.	–	
TIMP-2	w.c.	0.551***	w.c.	w.c.	w.c.	w.c.	0.428***	–
TNF-α	w.c.	0.341*	w.c.	w.c.	w.c.	w.c.	w.c.	w.c.
IL-1β	w.c.	w.c.	w.c.	w.c.	w.c.	w.c.	w.c.	w.c.
IL-6	w.c.	w.c.	w.c.	w.c.	0.348*	0.436**	0.291*	w.c.
IL-8	w.c.	w.c.	w.c.	w.c.	0.421**	0.308*	w.c.	w.c.
IL-10	w.c.	w.c.	w.c.	w.c.	w.c.	w.c.	0.311*	w.c.
IL-12	w.c.	w.c.	w.c.	w.c.	w.c.	w.c.	w.c.	w.c.
Fibrinogen	w.c.	w.c.	w.c.	0.279*	0.441***	0.442***	0.235*	w.c.
Leukocytes	0.198*	w.c.	w.c.	w.c.	0.210*	0.343***	w.c.	w.c.
Erythrocytes	w.c.	w.c.	w.c.	–0.227*	w.c.	w.c.	–0.225*	–0.227*
Thrombocytes	w.c.	w.c.	w.c.	w.c.	0.171*	0.113	0.311**	w.c.

Significant differences between individual biomarkers: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. w.c. = Weak correlation (<0.150).

Table 4. Combined sensitivity, specificity, PPV, and NPV for optimal cutoff points[1]

	AUC	95% CI	p value	p value[2]	Sensitivity	Specificity	PPV	NPV
MMP-1	0.63	0.53–0.73	0.0174	0.100	56.8	70.3	73.8	52.5
MMP-7	0.66	0.56–0.77	0.0005	0.016	86.5	50.0	86.5	50.0
TIMP-1	0.63	0.53–0.72	0.0220	0.049	89.2	37.5	85.7	45.2
TNF-α	0.63	0.47–0.77	0.1252	0.120	70.6	53.65	75.0	48.0
IL-8	0.65	0.49–0.79	0.0607	0.092	56.3	82.1	76.7	64.3
MMP-1 + MMP-7	0.70	0.602–0.788	0.0001	0.0028	78.4	60.9	83.0	53.7
MMP-1 + TIMP-1	0.67	0.573–0.764	0.0012	0.0009	73.0	62.5	80.0	52.9
MMP-7 + TIMP-1	0.69	0.599–0.786	0.0001	0.0006	67.6	70.3	78.9	56.8
MMP-1 + MMP-7 + TIMP-1	0.71	0.62–0.80	0.0001	0.0007	83.8	59.38	86.4	54.4
MMP-1 + MMP-7 + TIMP-1 + TNF-α	0.71	0.61–0.79	0.0001	0.0056	64.9	73.44	78.3	58.5
MMP-1 + MMP-7 + TIMP-1 + IL-8	0.71	0.61–0.79	0.0001	0.0014	86.5	67.19	89.4	60.1

[1] The optimal decision rule (cutoff point) for prediction of unstable carotid lesions was determined by ROC curve analysis using Youden index [J = maximum (sensitivity + specificity – 1)] for each factor with significant differences between the groups and their combinations [23]. AUC = Area under the curve; 95% CI = 95% confidence interval.

[2] Correction for multiple testing by using Benjamini and Hochberg approach.

Discussion

So far only few studies have evaluated the usefulness of multiple biomarkers for the possible prediction of cardiovascular disease, and up to date none was concerned with vulnerable carotid lesions. A recent investigation from the Framingham Heart Study in 3,209 participants demonstrated that the combination of various biomarkers enhanced the prediction of cardiovascular disease by 2-fold and even 4-fold regarding the enhanced risk of

death compared with a single biomarker [19]. Therefore, the aim of this study was to evaluate relevant biomarkers for vulnerability of carotid lesions and to combine them in a multimarker score to prove whether a set of such biomarkers might enhance the predictive value for patients with unstable plaques.

From the various MMPs (MMP-1, -2, -3, -7, -8, -9) and their inhibitors (TIMP-1, -2) that were analyzed in this study, serum levels of MMP-1, -7, and TIMP-1 were significantly increased in patients with unstable carotid lesions as shown by single comparisons between the groups. Furthermore, MMP-7 and TIMP-1 demonstrated significant differences also following correction for multiple testing. Both MMP-1 and -7 together with their inhibitor TIMP-1 play an important role in tissue remodeling, tissue degradation, progress and development of atherosclerotic lesions [7, 25]. MMP-1 is able to digest collagenous and noncollagenous matrix substrates and is believed to be responsible for the destabilization of the fibrous cap and plaque rupture [7, 26, 27]. MMP-7 is one of the most active enzymes degrading proteoglycans and may thus serve as a new potential biomarker of plaque instability. Vessel wall proteoglycans play a crucial role in the pathogenesis of atherosclerosis by trapping and storing different growth factors, lipoproteins, cholesterol, and a variety of other components of extracellular matrix [27].

Interestingly, no statistically significant differences between patients with stable and unstable lesions were observed for MMP-2 and -9 as described before in other studies [13–15]. The reason for these discrepancies might be first that both Borrelli et al. [13] and Alvarez et al. [15] graded all plaques as type V or VI (advanced lesions according to AHA) with type V classified as stable and type VI as complicated and unstable. In contrast, we performed more detailed morphological and histological analysis of the carotid lesions in accordance with Virmani et al. [3, 23] and defined unstable plaques as those with a thin fibrous cap covering a large necrotic core. Thus, also patients with carotid lesion type VI (defined as type V with thrombotic deposits and/or marked hemorrhage) with a thick fibrous cap were considered as stable. Such a thrombus or hemorrhage was mainly anterior and apparently not the reason why the patients underwent the current CEA. Furthermore, patients with advanced carotid stenoses, independent of whether the plaque is stable or unstable, may have enhanced levels of MMP-2 or/and MMP-9. Interestingly, in accordance with previous reports [14, 15], MMP-9 was significantly increased in the subgroup of patients with neurological symptoms (symptomatic patients, data not shown) compared with asymptomatic individuals. This would support the hypothesis that MMP-2 and MMP-9 might be more associated with neurological symptoms but are too unspecific or not sensitive enough to detect plaque vulnerability.

From the inflammatory factors such as fibrinogen and CRP, TNF-α, IL-1β, -6, -8, -10, -12 only TNF-α and IL-8 revealed significantly increased blood levels in patients with unstable versus stable carotid lesions by using single comparisons between the groups. TNF-α is one of the main proinflammatory cytokines derived from endothelial and smooth muscle cells as well as macrophages and stimulates the synthesis of a plethora of other inflammatory factors [13, 14, 28]. IL-8 is a CXCL8 cytokine chemoattractive for T cells and has proatherogenic action by stimulating the secretion of IFN-γ and macrophage accumulation [29]. This was apparently the reason for the relatively strong correlation between IL-8 in the blood of study patients and the number of macrophages within the carotid plaques ($r = 0.505$). Interestingly, no significant differences were found for CRP, IL-1β or IL-6, also known as main contributors of inflammation in atherosclerotic plaques [10]. However, it has to be considered that all patients included in the study had already advanced carotid stenosis of types V–VIII. In these patients, atherosclerotic lesions are frequently accompanied by chronic inflammation. Furthermore, significantly enhanced levels of CRP were observed in diseased patients compared to healthy individuals (CRP, unstable/stable vs. healthy: 1.35/1.05 vs. <0.5 mg/dl). Thus, some inflammatory factors may commonly correlate with advanced carotid stenosis or neurological symptoms being, however, not specific enough to detect vulnerable lesions [30].

The main aim of the study was, however, to assess whether a combination of relevant biomarkers of advanced carotid lesions might improve the prediction of lesion vulnerability compared with single biomarkers. Various combinations of the above-described biomarkers were analyzed by ROC. The best predictive value was found for the combination of MMP-1, -7, TIMP-1, and IL-8 with up to 90% of patients correctly diagnosed as individuals with unstable plaques and up to 60% of individuals correctly diagnosed as negative (table 4). It has to be emphasized that both PPV and NPV are important for the correct determination of vulnerable plaques. In our study, the combination of various biomarkers increased not only the possible prediction of vulnerable plaques but also the exclusion of patients with stable lesions. Furthermore, the use of combined biomarkers increased the sig-

nificance values between the groups compared to the significance of single factors. So using a combination of more than one biomarker for the prediction of unstable carotid plaques, significant differences were observed also following correction for multiple testing. These results confirm our assumption that multiple analyses of relevant biomarkers can improve the overall predictive value and reduce the risk of inaccurate diagnosis.

Limitation of the Study. Despite the careful and detailed characterization of all carotid plaques, some patients with small or unremarkable ruptures or fissures could still escape our attention. However, due to the exclusion of patients without well-defined plaque morphology, such mistakes were reduced to a minimum. Furthermore, our study is limited by the relatively small sample size for exact determination of truly appropriate cutoff values of each individual biomarker. The use of such cutoff values for the prediction of vulnerable carotid plaques in the clinical routine requires further multicentric studies with larger patient groups, which we intend to do in the future.

In summary, we performed a detailed multiple analysis of various predictive biomarkers in patients with advanced carotid stenosis to detect vulnerable lesions for each single individual. Using a multimarker score, ROC analysis and the estimated cutoff values we demonstrated the usefulness of a set of relevant biomarkers that play a role in plaque instability as a possible predictor of ischemic stroke. Larger clinical studies with enhanced statistical power are necessary to prove the relevance of the biomarkers found in our study and to find further prognostic markers to generate a set of reliable biomarkers for identifying vulnerable plaques in an attempt to prevent stroke.

Acknowledgments

The authors would like to thank Renate Hegenloh for her technical competence and help. Financial support for this study was partially provided by the Jung Foundation.

References

1 Bamford J, Sandercock P, Dennis M, Burn J, Warlow C: Classification and natural history of clinically identifiable subtypes of cerebral infarction. Lancet 1991;337:1521–1526.
2 Rothwell PM, Gutnikov SA, Warlow CP: Reanalysis of the final results of the European Carotid Surgery Trial. Stroke 2003;34:514–523.
3 Virmani R, Burke AP, Farb A, Kolodgie FD: Pathology of the vulnerable plaque. J Am Coll Cardiol 2006;47:C13–C18.
4 Chalela JA: Evaluating the carotid plaques: going beyond stenosis. Cerebrovasc Dis 2009;27(suppl 1):19–24.
5 Wahlgren CM, Zheng W, Shaalan W, Tang J, Bassiouny HS: Human carotid plaque calcification and vulnerability: relationship between degree of plaque calcification, fibrous cap inflammatory gene expression and symptomatology. Cerebrovasc Dis 2009;27:193–200.
6 Moreno PR, Purushothaman KR, Sirol M, Levy AP, Fuster V: Neovascularization in human atherosclerosis. Circulation 2006;113:2245–2252.
7 Newby AC: Dual role of matrix metalloproteinases (matrixins) in intimal thickening and atherosclerotic plaque rupture. Physiol Rev 2005;85:1–31.
8 Sundström J, Vasan RS: Circulating biomarkers of extracellular matrix remodeling and risk of atherosclerotic events. Curr Opin Lipidol 2006;17:45–53.
9 Ferri N, Paoletti R, Corsini A: Biomarkers for atherosclerosis: pathophysiological role and pharmacological modulation. Curr Opin Lipidol 2006;17:495–501.
10 Lind L: Circulating markers of inflammation and atherosclerosis. Atherosclerosis 2003;169:203–214.
11 Wu JT, Wu LL: Linking inflammation and atherogenesis: soluble markers identified for the detection of risk factors and for early risk assessment. Clin Chim Acta 2006;366:74–80.
12 Takahashi W, Ohnuki T, Ohnuki Y, Kawada S, Takagi S: The role of high-sensitivity C-reactive protein in asymptomatic intra- and extracranial large artery diseases. Cerebrovasc Dis 2008;26:549–555.
13 Borrelli V, di Marzo L, Sapienza P, Colasanti M, Moroni E, Cavallaro A: Role of platelet-derived growth factor and transforming growth factor beta1 in the regulation of metalloproteinase expressions. Surgery 2006;140:454–463.
14 Turu MM, Krupinski J, Catena E, Rosell A, Montaner J, Alvarez-Sabin J, Cairols M, Badimon L: Intraplaque MMP-8 levels are increased in asymptomatic patients with carotid plaque progression on ultrasound. Atherosclerosis 2006;187:161–169.
15 Alvarez B, Ruiz C, Chacon P, Alvarez-Sabin J, Matas M: Serum values of MMP-2 and MMP-9 as related to unstable plaque and inflammatory cells in patients with greater than 70% carotid artery stenosis. J Vasc Surg 2004;40:469–475.
16 Sapienza P, di Marzo L, Borrelli V, Sterpetti AV, Mingoli A, Piagnerelli R, Cavallaro A: Basic fibroblast growth factor mediates carotid plaque instability through metalloproteinase-2 and -9 expression. Eur J Vasc Endovasc Surg 2004;28:89–97.
17 Molloy KJ, Thompson MM, Schwalbe EC, Bell PR, Naylor AR, Loftus IM: Elevation in plasma MMP-9 following carotid endarterectomy is associated with particulate cerebral embolisation. Eur J Vasc Endovasc Surg 2004;27:409–413.
18 Bicknell CD, Peck D, Lau NM, Alkhamesi NA, Cowling MG, Clark MW, Jenkins MP, Wolfe JH, Darzi AW, Cheshire NJ: The relationship between plasma MMP-1, -7, -8 and -13 levels and embolic potential during carotid endoluminal intervention. Eur J Vasc Endovasc Surg 2004;28:500–507.
19 Thakore AH, Guo CY, Larson MG, Corey D, Wang TJ, Vasan RS, D'Agostino RB Sr, Lipinska I, Keaney JF Jr, Benjamin EJ, O'Donnell CJ: Association of multiple inflammatory markers with carotid intimal medial thickness and stenosis (from the Framingham Heart Study). Am J Cardiol 2007;99:1598–1602.

20 Wang TJ, Gona P, Larson MG, Tofler GH, Levy D, Newton-Cheh C, Jacques PF, Rifai N, Benjamin EJ, D'Agostino RB, Vasan RS: Multiple biomarkers for the prediction of first major cardiovascular events and death. N Engl J Med 200;355:2631–2639.
21 Addabbo F, Mallamaci F, Leonardis D, Tripepi R, Tripepi G, Goligorsky MS, Zoccali C: Searching for biomarker patterns characterizing carotid atherosclerotic burden in patients with reduced renal function. Nephrol Dial Transplant 2007;22:3521–3526.
22 Stary HC: Natural history and histological classification of atherosclerotic lesions: an update. Arterioscler Thromb Vasc Biol 2000; 20:1177–1178.
23 Virmani R, Kolodgie FD, Burke AP, Farb A, Schwartz SM: Lessons from sudden coronary death: a comprehensive morphological classification scheme for atherosclerotic lesions. Arterioscler Thromb Vasc Biol 2000; 20:1262–1275.
24 Benjamini Y, Hochberg T: Controlling the false discovery rate: a practical and powerful approach to multiple testing. J R Stat Soc B 1995;85:289–300.
25 Dollery CM, Libby P: Atherosclerosis and proteinase activation. Cardiovasc Res 2006; 69:625–635.
26 Lemaitre V, D'Armiento J: Matrix metalloproteinases in development and disease. Birth Defects Res C Embryo Today 2006;78: 1–10.
27 Wu YW, Yang WS, Chen MF, Lee BC, Hung CS, Liu YC, Jeng JS, Huang PJ, Kao HL: High serum level of matrix metalloproteinase-1 and its rapid surge after intervention in patients with significant carotid atherosclerosis. J Formos Med Assoc 2008;107:93–98.
28 Wight TN, Merrilees MJ: Proteoglycans in atherosclerosis and restenosis: key roles for versican. Circ Res 2004;94:1158–1167.
29 Libby P, Sukhova G, Lee RT, Galis ZS: Cytokines regulate vascular functions related to stability of the atherosclerotic plaque. J Cardiovasc Pharmacol 1995;25(suppl 2):S9–S12.
30 Ito T, Ikeda U: Inflammatory cytokines and cardiovascular disease. Curr Drug Targets Inflamm Allergy 2003;2:257–265.