MMP and TIMP Alterations in Asymptomatic and Symptomatic Severe Recurrent Carotid Artery Stenosis

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Abstract  Objectives: This study aimed to determine whether the plasma levels of matrix metalloproteinases (MMPs)-2 and -9 and their specific inhibitors (tissue inhibitors of metalloproteinases (TIMPs-1 and -2)) were altered in patients with symptomatic and asymptomatic, severe, recurrent carotid artery stenosis.

Patients: Fifty-two patients (out of a total of 621) who had undergone successful carotid artery endarterectomy (CEA) between 1999 and 2003 and developed recurrent carotid artery stenosis (>70%) were included in the study. Restenosis was symptomatic in 23 patients and asymptomatic in 29 patients.

Methods: Recurrent carotid artery stenosis was classified based on presentation, and as early–intermediate (6 months to 3 years) or late (>3 years). A detailed clinical history was taken and two blood samples were drawn from each patient to determine plasma levels of MMPs and TIMPs along with other biological parameters. Recurrent stenosis was confirmed with computed tomographic angiography.

Results: Patients with symptomatic restenosis had significantly (p < 0.001) higher active MMP-2 and -9 plasma values and significantly (p < 0.001) lower TIMP-1 and -2 plasma values when compared to patients with asymptomatic restenosis. Plasma concentrations of active MMPs were higher and TIMPs lower in patients affected with late recurrent stenosis as compared to early–intermediate restenosis (p < 0.001). No differences were recorded in latent MMP plasma values. Multivariate analysis showed that active MMP-2 and -9 were independent predictors of late recurrent carotid artery stenosis (p < 0.03 and p < 0.001, respectively).

Conclusions: Higher plasma concentrations of active MMP-2 and -9 were associated with an increased risk of carotid restenosis with plaque recurrence.

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The incidence of recurrent stenosis after carotid endarterectomy (CEA) varies from 10% to 25%,1–3 but symptoms occur in 0.6–3.6% of patients.4–6 Previous studies have concluded
that the natural history of recurrent carotid stenosis is generally benign.5,7 Present data indicate that 15 years after a successful CEA, one-third of the patients can be expected to develop recurrent carotid artery disease, one-half of whom can be expected to suffer from a recurrent ischaemic event.8,9 Although no true consensus exists with regards to the natural history of these lesions, and their treatment remains controversial, re-operation is considered necessary in 1–8% of cases.1,10 It is accepted that re-operation for significant recurrent carotid artery stenosis is indicated for patients with symptomatic disease, but several authors have also recommended re-operation for asymptomatic patients with >80% carotid artery restenosis.3

The timing and symptoms of carotid artery restenosis are of biological and clinical importance. Early restenosis is marked by the accumulation of smooth muscle cells and fibrous tissue and late recurrence by increased macrophage infiltration, calcification and a lipid core, thus resembling primary plaques.11 Both early and late symptomatic restenotic plaques are associated with high macrophage infiltration and a large lipid core.11 These processes involve extensive reorganisation of the extracellular matrix, which is regulated through a balance between matrix metalloproteinases (MMPs) and the tissue inhibitor of metalloproteinases (TIMPs). MMPs — a family of zinc-containing enzymes, secreted as inactive precursors — can degrade the components of the vascular extracellular matrix.12,13 MMP-2 and -9 belong to the MMP gelatinase family and efficiently degrade type IV collagen — the major structural component of the basement membrane. Endogenous TIMPs regulate MMP activity.12 TIMP-1 inhibits the activity of all the active MMPs, with a particular affinity for MMP-1, -2, -3 and -9,14 and it is the more widely distributed of the TIMPs. TIMP-2 selectively limits MMP-2 activity.14

This study tested the hypothesis that alterations in plasma MMP-2 and -9 and their specific inhibitors (TIMP-1 and -2) during follow-up may predict risk for severe recurrent carotid artery stenosis.

**Patients and Methods**

Between January 1999 and December 2003, 621 consecutive patients (526 men and 95 women; mean age: 69 ± 9 years; median age: 68 years; age range: 56–86 years) presenting at our institution with internal carotid artery stenosis (≥70%) underwent CEA. All primary operations were performed in a standard fashion under general anaesthesia with electroencephalographic monitoring.

This research was approved by the Committee on Research Ethics at our institution, in accordance with the Declaration of Helsinki of the World Medical Association. All patients included in the study gave informed, written consent.

The patients were followed up at 4 and 6 months postoperatively, and at 6-month intervals thereafter, by B-mode ultrasonography and colour imaging (Acuson 128 XP/4 with a 7.5 MHz probe; Acuson Corp., Mountain View, CA, USA). B-mode ultrasonography and colour imaging were used to assess the presence of recurrent stenosis. The peak systolic velocities of the internal carotid artery >230 cm s⁻¹ were used to diagnose haemodynamically significant stenosis ≥70%.15 An end-diastolic velocity >100 cm s⁻¹ was also used when clinical or technical factors aroused concern that the internal carotid artery peak systolic velocity may not be representative of the extent of disease.15 The recurrent stenotic plaque was defined as uniformly anechogenic, predominantly hypo-echoic, predominantly echogenic or uniformly echogenic, according to the Gray-Weale scale. The luminal surface of the plaque was also defined as regular, irregular with a recess between 0.4 and 2 mm in depth and width or irregular with a recess >2 mm in depth and width. Recurrent stenosis was considered to be present only if the abnormality detected by B-mode ultrasonography and colour imaging was not detected on the first immediate postoperative examination and if it persisted for at least two examinations performed within 6 months of the original examination. B-mode ultrasonography and colour-imaging findings consistent with ≥70% stenosis were validated with computed tomographic angiography (CTA) according to the standards proposed by the North American Symptomatic Carotid Endarterectomy Trial (NAS-CT).16 Briefly, CTA axial images, both contrast and non-contrast, were used for measurements. Coronal and sagittal multiplanar reformats were used to identify the carotid orientation to ensure true cross-sectional measurements in all of the evaluated arteries. These measurements were verified with measures from reformats to ensure accuracy in obtaining the narrowest diameter in a true cross-sectional plane. Special attention to calcified high-density regions of the plaque was reinforced with extra-windowing and contrast modification.

In the presence of recurrent carotid artery stenosis >70%, a complete history was taken, and physical and neurological examinations were carried out by the neurologists. Focal cerebral ischaemic events were defined as a transient ischaemic attack (TIA), amaurosis fugax, central retinal artery occlusion and minor (defined as a clinical syndrome of rapidly developing signs or symptoms of focal loss of cerebral function of vascular origin lasting >24 h and not leading to a handicap or significant impairment (modified Rankin scale <3)17 in the activities of daily living) and major stroke (defined as a clinical syndrome of rapidly developing signs or symptoms of focal loss of cerebral function of vascular origin lasting >24 h and leading to a handicap or significant impairment (modified Rankin scale >3)17 in the activities of daily living). The patients with recurrent carotid stenosis were then stratified into asymptomatic and symptomatic groups and, based on the recurrence interval, the recurrent carotid artery stenosis was classified as early—intermediate (6 months to 3 years) or late (>3 years).

At carotid artery restenosis confirmation, or within a median period of 8 days (mean: 7 ± 4 days; median: 8 days; range: 4–16 days) after a focal cerebral ischaemic event, two blood samples were drawn from each patient to determine plasma levels of MMPs and TIMPs and other biological parameters. Risk factors and associated diseases were also documented for each patient, along with the use of aspirin (100 mg per day), ticlopidine (250 mg per day) or lipid-lowering drugs. The risk factors included tobacco use, diabetes (insulin-dependent or -independent), cardiac disease (prior myocardial infarction, stable or unstable
angina or ST-segment alteration on electrocardiogram), hypertension (diastolic blood pressure: >85 mmHg), renal disease (blood urea nitrogen: >7.1 mmol l⁻¹; creatinine level: >266 μmol l⁻¹; creatinine clearance: <50 ml per min), pulmonary disease (PO₂: <60 mmHg; PCO₂: >50 mmHg; pulmonary function tests: <80% of predicted) and dyslipidaemia (total cholesterol level: >5 mmol l⁻¹, low-density lipoprotein level: >4 mmol l⁻¹, high-density lipoprotein level: <1 mmol l⁻¹ and non-fasting triglyceride level: >1.7 mmol l⁻¹). Associated diseases were represented by obesity (weight body: >20% of ideal) and an alteration in the coagulation assay. The concomitant presence of peripheral vascular and/or aneurysmal diseases in the patients recruited into this study was screened with ankle–brachial index (ABI) measurements and abdominal aortic ultrasound.

A control group of 20 age-matched healthy volunteers (mean age: 65 ± 5 years; median age: 66 years; age range: 47–79 years) without atherosclerotic lesions (excluded by carotid and aortic ultrasonography and ABI measurements) served as a reference for biological parameters.

Serum matrix metalloproteinase-2 and -9 and their specific inhibitor activity and immunoassays

Plasma was obtained from EDTA-anticoagulated peripheral blood samples centrifuged at 4000 x g for 10 min, and stored at −70 °C for further analysis. Endogenous plasma MMP-2 and -9 were assessed using the Biotrak Activity Assay System (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK). This system measures latent enzymes (pro-form and active forms, but with a relatively low affinity for TIMP-bound enzymes, as reported by the manufacturer) after activation using p-aminophenylmercuric acetate, wherein the exclusion of p-aminophenylmercuric acetate results in a measurement of the endogenous free active MMP-2 and -9 fractions. Total MMP-2 and -9 and TIMP-1 and -2 were determined in EDTA-plasma samples using an enzyme-linked immunosorbent assay (ELISA) technique (Amersham Pharmacia), estimating the ratio of pro-MMP-2 and -9 (or latent) to active enzyme, therefore, indicating the degree of zymogen activation. The average coefficient of variance for both activity and ELISA assays was <5.5%.

Statistical analysis

Data were analysed with a computer software program (SPSS, version 12.0, for Windows, Basic and Advanced Statistics, 1989–2003; SPSS Inc., Chicago, IL, USA). Due to the sample sizes, non-parametric tests were applied. Continuous variables were analysed with either the Mann–Whitney U test or Kruskal–Wallis ANOVA, followed by the Bonferroni post hoc test calculated dividing the p value (0.05) by the number of paired comparisons made, thus adjusting for a total of 24 comparisons. Stepwise, logistic regression analysis adjusted for demographics (age and gender), medical co-morbid conditions (active tobacco use, hypertension, diabetes, ischaemic heart disease, renal disease, pulmonary disease, obesity, coagulopathy, total cholesterol level, LDL-cholesterol level, HDL-cholesterol level and non-fasting triglyceride level) and medical treatments (angiotensin-converting enzyme inhibitors, Ca²⁺ antagonists, β-blockers, nitrates, lipid-lowering drugs, aspirin and ticlopidine) was used to test the association between plasma active/latent MMP-2 and -9 and TIMP-1 and -2 and recurrent carotid artery stenosis presentation. Odds ratios (ORs) are expressed with 95% confidence intervals (CI) in this analysis. All results are expressed as the mean ± standard deviation. Differences with α-level of <0.05 were considered statistically significant.

Results

This study included 389 (63%) patients who underwent surgery for symptomatic disease and a further 232 (37%) patients for asymptomatic disease. Overall, 44 (7%) patients had major complications (stroke and/or
myocardial infarction) or died during the 30-day postoperative period: 36 (9%) symptomatic patients and eight (3%) asymptomatic patients. During follow-up (mean: 74 ± 9 months; median: 73 months; range: 60–108 months), 15 (2%) patients were lost, 29 (5%) patients died secondary to ipsilateral stroke, 42 (7%) patients died of unrelated causes, 11 (2%) patients developed recurrent carotid artery stenosis ranging between 50% and 69% and 52 (9%) patients developed recurrent carotid artery stenosis ≥70% (mean: 74 ± 6%; median: 77%; range: 70–99%) at a mean follow-up of 45 ± 13 months (median: 42 months; range: 24–96 months).

The latter group was eligible for further analysis. No other clinical vascular diseases (i.e., abdominal aortic aneurysm and peripheral vascular disease) affected the study group. Symptomatic patients had no significant differences (p = NS) in demographics, risk-factor-associated diseases and medical treatments. The demographics, clinical and biological characteristics of the series are depicted in Table 1. Among the patients who presented with symptoms, 12 had a TIA, six had amaurosis fugax, four had central retinal artery occlusion and one had a minor stroke.

### Plasma MMP-2 and -9 and TIMP-1 and -2 levels

Plasma values of both active and latent MMP-2 (20 ± 5 ng ml⁻¹ and 902 ± 34 ng ml⁻¹, respectively) and -9 (5 ± 2 ng ml⁻¹ and 74 ± 3 ng ml⁻¹, respectively) of age-matched healthy volunteers were significantly lower (p < 0.001) compared to the other groups. On the other hand, plasma values of TIMP-1 (1234 ± 112 ng ml⁻¹) and -2 (190 ± 13 ng ml⁻¹) were significantly higher (p < 0.001) as compared to the other groups.

Table 2 summarises the active and latent MMPs and TIMPs plasma levels in patients affected with recurrent carotid artery stenosis according to symptoms and recurrence interval. Symptomatic patients had a significantly higher elevation of active MMP-2 and -9 plasma values as compared to asymptomatic patients. These alterations were influenced by the interval of recurrence (early–intermediate or late).

Plasma values of active MMPs were, in fact, significantly higher in patients affected with late recurrent stenosis as compared to early–intermediate restenosis. No significant alterations were recorded for latent MMP release. Symptomatic patients had significantly lower plasma values of TIMP-1 and -2, and these alterations were associated with the interval of recurrence. Plasma values of TIMPs were significantly lower in patients affected with late recurrent carotid artery stenosis as compared to early–intermediate restenosis.

After stepwise logistic regression analysis, active MMP-2 and -9 were the only variables that remained in the model as independent predictors of late recurrent carotid artery stenosis (Table 3).

### Discussion

Principally, our study found that, independent from its pathogenesis and clinical presentation, carotid plaque recurrence may be indicated by an elevation of MMPs, with late carotid artery restenosis having higher plasma MMPs than early–intermediate plaques. At an early stage of carotid artery restenosis, MMPs control extracellular matrix degradation and the release of matrix-degrading metalloproteinases, including MMP-2 and -9, which facilitate intimal remodelling. These lesions are similar to those reported in experimental studies of intimal hyperplasia in animal vascular injury models, in which a marked increase in matrix expression occurs even after cell

| Table 2 Plasma levels of active and latent MMPs and TIMPs in patients affected with recurrent carotid artery stenosis based on symptoms and recurrence interval |
|--------------|----------|--------------|----------|----------|
|              | Asymptomatic | Symptomatic |          |          |
|              | Early–intermediate | Late | Early–intermediate | Late |
| (n = 12) | (n = 17) | (n = 9) | (n = 14) |
| MMP-2 | 81 ± 19(a) | 219 ± 54(b) | 410 ± 54(c) | 910 ± 96(d) |
| Active | 1917 ± 333 | 1835 ± 465 | 1914 ± 272 | 1879 ± 270 |
| Latent | 11 ± 3(e) | 24 ± 8(f) | 40 ± 6(g) | 87 ± 10(h) |
| MMP-9 | 54(c) | 910 ± 96(d) | 90 ± 71(i) | 13 ± 7(j) |

All values are expressed as ng ml⁻¹ ± SD (standard deviation).

Significance: (a) vs. (b), (a) vs. (c), (a) vs. (d), (a) vs. (e), (a) vs. (f); (a) vs. (g), (a) vs. (h), (a) vs. (i), (a) vs. (j); (b) vs. (c), (b) vs. (d), (c) vs. (d), (c) vs. (e), (c) vs. (f), (d) vs. (e), (d) vs. (f), (d) vs. (g), (d) vs. (h), (d) vs. (i), (d) vs. (j); (e) vs. (f), (e) vs. (g), (e) vs. (h), (e) vs. (i), (e) vs. (j); (f) vs. (g), (f) vs. (h), (f) vs. (i), (f) vs. (j); (g) vs. (h), (g) vs. (i), (g) vs. (j); (h) vs. (i), (h) vs. (j); (i) vs. (j).

### Table 3 Biomarkers associated with late recurrent carotid artery stenosis in a stepwise logistic regression model

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>OR (95% CI)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Active MMP-2</td>
<td>1.013 (1.004–1.037)</td>
<td>0.003</td>
</tr>
<tr>
<td>Active MMP-9</td>
<td>1.053 (1.030–1.099)</td>
<td>0.001</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>0.989 (0.974–1.003)</td>
<td>NS</td>
</tr>
<tr>
<td>TIMP-2</td>
<td>0.992 (0.983–1.011)</td>
<td>NS</td>
</tr>
</tbody>
</table>

NS = not significant.
proliferation has subsided. For carotid artery restenotic lesions that are similar to primary carotid plaques, the release of MMPs increases with a simultaneous decrease of TIMPs.

In the presence of symptomatic restenosis, the plasma levels of MMPs increased even more and those observed in late symptomatic carotid artery restenosis doubled when compared to early–intermediate symptomatic carotid artery restenosis. Conversely, the release of the specific inhibitors was higher in early–intermediate lesions compared to late recurrence. Although the number of patients included in our study was limited – thus diminishing the strength of our conclusions, at least in part – stepwise logistic regression analysis confirmed the strong independence from the other confounders of plasma active MMP-2 and -9 as risk indicators for late recurrent carotid artery stenosis, thus supporting our previous observation in primary carotid artery stenosis.

We hypothesise that growth factors and cytokines, which are also present in restenotic lesions, may up-regulate the release of MMPs and down-regulate the release of TIMPs, as occurs in primary atherosclerotic carotid plaques. In our previous studies, we demonstrated that in primary atherosclerotic carotid plaques basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF) and tumour growth factor (TGF-β1) are responsible for the dysregulation in the release, activity and expression of MMPs and TIMPs. We believe that the interactions between growth factors, cytokines and MMPs and their natural inhibitors may provoke an unstable restenotic plaque either in the early–intermediate or late period.

The clinical importance of our study lies in the fact that, in the future, plasma-level alterations of MMPs may signal the need for re-intervention in order to reduce the risk of recurrent cerebral ischaemia. However, much further research and validation is necessary before these biomarkers can be proposed as specific and sensitive tests.

At present, no conclusive data are present in the literature with respect to MMP and TIMP alterations. In some studies, MMP markers seem to be able to detect unstable plaques or intra-stent stenosis, but, in others, MMPs are not specific and sensitive tests, with plasma levels being similar in symptomatic and asymptomatic patients. Furthermore, to be useful, these biomarkers should highlight the appropriate time-window in which an intervention would be efficacious.

A limitation of our study was that the time between symptom occurrence and blood analysis had a median of 8 days. This delay might have influenced our results. However, several authors have recently demonstrated that MMPs have a peak in the first 48–72 h after the onset of neurological symptoms and are strongly associated with neurological deterioration.

In conclusion, increases in plasma MMP-2 and -9 after CEA is associated with a higher risk of recurrent carotid artery stenosis, thus representing a potential future test for patient surveillance.

**Conflict of Interest**

None.

**Funding**

None.

**References**


