Serum matrix metalloproteinase-8, tissue inhibitor of metalloproteinase and myeloperoxidase in ischemic stroke

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ABSTRACT

Background and aims: Matrix metalloproteinase (MMP)-8 and myeloperoxidase (MPO) may contribute to cerebral damage in acute ischemic stroke. We tested the hypothesis that levels of MPO, MMP-8 and the ratio between MMP-8 and its regulator, tissue inhibitor of metalloproteinase (TIMP-1), are increased in acute ischemic stroke and its etiologic subgroups and they correlate with stroke severity.

Methods: In a cross-sectional case–control study, serum concentrations of MMP-8, MPO and TIMP-1 were assessed within 24 h after admission in 470 first-ever ischemic stroke patients and 809 age- and sex-matched controls, randomly selected from the population. Odds ratios (OR) per decade of log transformed dependent variables were calculated and adjusted for age, sex and vascular risk factors.

Results: Levels of MMP-8 (OR 4.9; 95% CI 3.4–7.2), MMP-8/TIMP-1 ratio (3.0; 2.2–4.1) and MPO (6.6; 4.0–11.0) were independently associated with ischemic stroke. MMP-8 levels differed between etiologic stroke subgroups (p = 0.019, ANOVA), with higher levels in cardioembolic stroke and stroke due to large vessel disease, and lower levels in microangiopathic stroke. MMP-8, MMP-8/TIMP-1 ratio and MPO (p < 0.001) concentrations showed positive associations with stroke severity independent of stroke etiology.

Conclusions: Concentrations of serum neutrophil markers are increased after ischemic stroke and associate with stroke severity and etiology. The value of these biomarkers in diagnostics and prognostics is worth being evaluated.

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1. Introduction

Stroke is one of the major causes of death and disability worldwide [1]. Demographic changes will lead to increasing numbers of stroke patients [2]. Optimizing stroke prevention and acute stroke care is mandatory. Systemic inflammation plays a key role in both stroke formation and immediate tissue damage post stroke [3]. Identification of specific proinflammatory biomarkers might give an opportunity to lower stroke risk and enhance acute stroke care.

We recently described higher serum concentrations of matrix metalloproteinase (MMP)-8 and myeloperoxidase (MPO) in patients with acute ischemic stroke compared to stroke-free controls [4]. MMP-8 or collagenase-2 is a catalytically competent endoprotease that can decisively process extracellular matrix components and non-matrix bioactive substrates causing tissue destruction and modulation of immunoresponses [5]. By producing hypochlorite (HOCl), MPO can not only oxidatively activate latent MMP-8 but also inactivate tissue inhibitor of matrix metalloproteinase (TIMP)-1 [6]. TIMP-1 is an important endogenous inhibitor of MMP-8 capable of binding to the active site of MMP-8 in an equimolar ratio to maintain the physiological conditions. Although some studies of serum MMP-8, MPO, and TIMP-1 in stroke patients exist, such observations are limited. In a population-based cohort, circulating MPO and TIMP-1 concentrations, but not MMP-8, were associated with incident stroke in a 13-year follow-up [7]. However, little is known on the association between these serum factors and...
stroke severity and stroke etiology [8]. Based on data from a preliminary case-control study, we tested the hypotheses that serum levels of MMP-8 along with its activator MPO and TIMP-1 inhibitor are elevated in patients with ischemic stroke (IS), and especially that they are associated with stroke severity.

2. Patients and methods

“GENESIS” is a cross-sectional case-control study comprising 470 first-ever ischemic stroke (FEIS) cases (40% women, mean age 66.5 ± 10.8 years; 60% men, age 65.5 ± 10.7 years) and 809 age- and sex-matched controls (41.8% women, 66.4 ± 11.1; 58.2% men, age 67.9 ± 9.5 years), randomly selected from the general population. This study was established within the framework of the Ludwigshafen Stroke Study (LuStS), a population-based stroke registry that started on January 1st, 2006. Detailed descriptions of the LuStS registry and the “GENESIS” study have been published recently [9,10]. The studies were approved by the ethics committee of the Landesärztekammer Rheinland-Pfalz (837.333.05/4991)).

2.1. Inclusion and exclusion criteria

Inclusion criteria of both cases and controls covered males and females between 20 and 80 years of age, permanent residency in the study area of the LuStS registry, Caucasian ethnicity and written informed consent to study participation. Additional inclusion criterion for cases was the diagnosis of a FEIS based on an acute neurological deficit lasting ≥24 h with no other cause than cerebral ischemia. All cases received a cerebral CT or MRI.

Exclusion criteria for cases were acute transient ischemic attack, intracerebral, subdural or subarachnoid hemorrhage and ischemic stroke due to cerebral trauma or brain malignancy. Exclusion criteria for both cases and controls included any previous stroke, myocardial infarction within past 90 days, dementia, severe aphasia, insufficient understanding of the German language or any other relevant communication barrier and severe disability that precluded interview participation.

2.2. Recruitment

For recruitment of controls, a random sample of Ludwigshafen residents was drawn from the population registry including name, age, sex and address. Subsamples were consecutively taken to match the age and sex distribution of cases. Those selected received invitation letters with detailed information on the study and request for their participation. The participation rate for controls was 46.6%. The cases included incident stroke cases from the LuStS registry. According to the study protocol only in-patients at the Klinikum Ludwigshafen were asked for participation in “GENESIS”. This group represents about 89% of all cases in LuStS. The participation rate for cases was 73.7%.

2.3. Data collection and laboratory tests

Cases and controls were interviewed by trained personnel using a standardized questionnaire. We collected data on age, sex, anthropometric measures, previous diseases, frequency of previous dentist visits as markers of health behavior, number of teeth, smoking habits, alcohol intake, physical activity, dietary patterns, and medication and social history. In both, cases and controls, blood pressure was measured after 5 min of resting, a 12-lead electrocardiogram and a Duplex-sonography of brain supplying arteries were performed. Venous blood samples were collected in cases and controls, immediately frozen and stored at −70 °C until processing. In all patients, venipuncture was performed within first 24 h after hospital admission. Serum inflammation marker concentrations were determined by commercial ELISA kits according to the manufacturer’s instructions. The precision of the analysis are expressed as coefficient of variation (CV) in percent. Precision for TIMP-1 (Amersham Biotrak, GE Healthcare, Buckinghamshire, UK) was 3.1%, for MPO (Immundiagnostik AG, Bensheim, Germany) 4.8%. The serum MMP-8 concentrations were determined by a time-resolved immunofluorometric assay (IFMA) as described previously, and the interassay coefficient of variation (CV)% was 7.3% [11].

For the calculation of MMP-8⁄TIMP-1 molar ratios, the concentrations were converted to molarity using MWs of 65 kDa and 28 kDa as described previously [12]. Leucocyte count (XE analyserXE-2100; Sysmex) was determined shortly after admission.

2.4. Definition of variables

Cardiovascular risk factors were defined according to current national and international guidelines and have been described in detail [13]. Etiological subtypes of IS were ascertained using modified TOAST criteria (Trial of ORG 10172 in Acute Stroke Treatment) as described recently [13]. Stroke severity was measured by the National Institutes of Health Stroke Scale (NIHSS) on admission, as well as by the modified Ranking Scale (mRS) [14,15].

2.5. Statistical analysis

The Chi-squared (X²) test was used to compare categorical data. The t-test was applied to analyse normally distributed continuous variables. The Box-Cox method was used to check the compliance of each laboratory parameter with the normal distribution, taking into account the group influence (case-control). This method assesses the optimal transformation for the parameters to achieve normal distribution. If necessary, parameters were log transformed prior to multivariate analysis. All biomarkers of inflammation were additionally adjusted for cardiovascular risk-factors and parameters significantly different between cases and controls by univariate analysis using conditional multiple logistic regression analysis, stratified for the matching parameters sex and age. In the multivariate logistic model it was impossible to include the quotient MMP-8⁄TIMP-1 molar ratio. This quotient is a direct derivative of two predictors already included in the model, and would result in collinearity. We displayed the univariate statistics just for purpose of comparability with literature. Logistic regression analysis was used to calculate odds ratios (OR) for stroke with 95% confidence intervals (CI). To assess influence of IV-treatment with t-PA, we additionally performed sensitivity analysis excluding t-PA treated cases. Investigation of the influence of stroke etiology and NIHSS on admission on biomarkers of inflammation was performed by analysis of variance (ANOVA) complemented by Dunn’s procedure for multiple comparisons, which corrects for potential bias by multiple testing. NIHSS was separated into 4 classes: 0—2; 3—4; 5—9; >9. Influence of stroke etiology and severity was investigated in restriction to stroke cases. All tests were two-sided and level of significance was set to 5%. Data were analyzed using the software package SAS 9.4, and SAS JMP 1.2.

3. Results

Distribution of baseline characteristics is shown in Table 1, as previously published [10]. Patients were significantly more often diagnosed as having hypertension, diabetes mellitus, hyperlipidemia, coronary artery disease (CAD), myocardial infarct, chronic heart failure, peripheral artery disease (PAD), and atrial fibrillation (AF). They were more often physically inactive and more frequently current smokers. Compared to the control group, the number of
teeth was considerably lower in cases. Leucocyte count was significantly higher in cases. Table 2 presents mean serum MMP-8, MPO and TIMP-1 concentrations as well as MMP-8/TIMP-1 molar ratio in patients and controls. All parameters showed significant differences in univariate and multivariate analysis. They all were significantly higher in the case group as compared to controls. Sensitivity analysis leaving out 48 cases that had received iv t-PA treatment did not notably change the results (Table 3).

The serum levels of inflammatory markers are summarized by NIHSS on admission in Fig. 1A. Analysis of variance showed significant associations between stroke severity and serum levels of MMP-8 ($p < 0.001$), MPO ($p < 0.001$) and MMP-8/TIMP-1 molar ratio ($p < 0.001$) but not TIMP-1 ($p = 0.5$) with higher values being measured in patients with more severe stroke.

Levels of inflammatory markers on admission are shown by stroke etiology in Fig. 1B. Analysis of variance showed significant differences between stroke subgroups for MMP-8 ($p = 0.019$) and MMP8/TIMP-1 ratio ($p = 0.047$), but not for MPO ($p = 0.113$) and TIMP-1 ($p = 0.071$). In patients with stroke due to small vessel disease (SVD), MMP-8 serum levels were significantly lower compared to patients with stroke due to large-vessel disease (LVD) or cardioembolism (CE). When analysing stroke etiology and categorized stroke severity in the ANOVA model simultaneously, only stroke severity remained significant ($p = 0.009$) while stroke etiology turned non-significant ($p = 0.241$).

### Table 2

Baseline characteristics of cases and controls.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Cases (n = 470)</th>
<th>Controls (n = 809)</th>
<th>$p$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age mean (SD)</td>
<td>65.9 (10.7)</td>
<td>67.3 (10.2)</td>
<td>0.03</td>
</tr>
<tr>
<td>Female sex n (%)</td>
<td>188 (40.0%)</td>
<td>338 (41.8%)</td>
<td>0.5</td>
</tr>
<tr>
<td>Hypertension n (%)</td>
<td>407 (86.6%)</td>
<td>536 (66.3%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Diabetes n (%)</td>
<td>145 (30.9%)</td>
<td>138 (17.1%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Hypcholesterolemia n (%)</td>
<td>342 (72.8%)</td>
<td>525 (64.9%)</td>
<td>0.004</td>
</tr>
<tr>
<td>Atrial fibrillation n (%)</td>
<td>92 (19.6%)</td>
<td>75 (9.3%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>History of myocardial infarct n (%)</td>
<td>66 (14.9%)</td>
<td>14 (1.7%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Coronary artery disease n (%)</td>
<td>99 (21.1%)</td>
<td>89 (11.0%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Congestive heart failure n (%)</td>
<td>60 (12.8%)</td>
<td>48 (5.9%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Periperal artery disease n (%)</td>
<td>46 (9.8%)</td>
<td>17 (2.1%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Physical activity n (%)$^b$</td>
<td>150 (31.9%)</td>
<td>447 (55.3%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Current smoking n (%)</td>
<td>145 (30.9%)</td>
<td>118 (14.6%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>High alcohol consumption n (%)$^c$</td>
<td>32 (6.8%)</td>
<td>47 (5.8%)</td>
<td>0.48</td>
</tr>
<tr>
<td>Number of teeth (SD)</td>
<td>12.5 (11.0)</td>
<td>15.5 (11.1)</td>
<td>&lt;0.001$^d$</td>
</tr>
<tr>
<td>Leucocyte count ($\times 1000$/mm$^3$) mean (SD)</td>
<td>8.3 (2.1)</td>
<td>6.7 (1.8)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

$p$-value refers to $X^2$-test. If $^a$ is shown, the $t$-test has been used; $^b$ leisure time physical activity that causes sweating $\geq$1/week; $^c$ > 50 g alcohol intake/day.

### 4. Discussion

In this large, carefully designed case-control study of ischemic stroke, we found that serum MMP-8, MPO, and TIMP-1 concentrations were strongly associated with acute ischemic stroke. While MMP-8, MPO, and MMP-8/TIMP-1 molar ratio were positively associated with stroke severity, serum levels of MMP-8 differed significantly between stroke subtypes.

MMPs play an important role in the cascade leading to brain injury after an ischemic stroke. MMP-1, -2, -3, -8, -9, -10, -13, and TIMP-1 are overexpressed in the infarcted brain tissue of stroke patients compared to non-ischemic areas [16]. Their sources are activated or infiltrating inflammatory cells such as microglia, macrophages, and neutrophils, and they contribute to the neurovascular perturbations comprising blood-brain barrier leakage and inflammatory reactions. Therefore, MMPs have been suggested as therapeutic targets for stroke. However, since MMPs are needed in the angiogenesis during the recovery, therapeutic inhibition of their activity needs to be carefully timed. The most studied MMP in CVD is MMP-9, but considering the potential of MMPs in treatments, the research should be broadened to other MMP family members.

MMP-8 is seldom studied specifically in stroke patients [4,17]. In our study, MMP-8 was directly associated with more severe stroke as measured by NIHSS. We did not analyze infarct volume, but stroke severity measured by NIHSS is closely related to it [18].

### Table 2

MMP-8, TIMP-1, MPO and MMP-8/TIMP-1-molar ratio in cases and controls.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Univariate$^a$</th>
<th>Multivariate per sero-parameter$^b$</th>
<th>Multivariate all 3 sero-parameters$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cases</td>
<td>Controls</td>
<td>OR (95% CI)</td>
</tr>
<tr>
<td>MMP-8 [ng/ml] mean$^d$ (95% CI)</td>
<td>58.3 (53.2–63.8)</td>
<td>24.2 (22.7–25.8)</td>
<td>8.2 (6.0–11.3)</td>
</tr>
<tr>
<td></td>
<td>N = 466</td>
<td>N = 803</td>
<td></td>
</tr>
<tr>
<td>TIMP-1 [ng/ml] mean$^d$ (95% CI)</td>
<td>127 (123–132)</td>
<td>122 (119–124)</td>
<td>3.5 (1.6–8.0)</td>
</tr>
<tr>
<td></td>
<td>N = 465</td>
<td>N = 803</td>
<td></td>
</tr>
<tr>
<td>MMP-8/TIMP-1 mean$^d$ (95% CI)</td>
<td>0.19 (0.17–0.21)</td>
<td>0.08 (0.08–0.09)</td>
<td>4.5 (3.4–5.5)</td>
</tr>
<tr>
<td></td>
<td>N = 465</td>
<td>N = 803</td>
<td></td>
</tr>
<tr>
<td>MPO [ng/ml] mean$^d$ (95% CI)</td>
<td>214 (201–227)</td>
<td>130 (124–137)</td>
<td>10.1 (6.6–15.4)</td>
</tr>
<tr>
<td></td>
<td>N = 466</td>
<td>N = 802</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Means and confidence limits have been calculated under log-transformation, and have been back-transformed to original scale; OR was calculated per decade of the dependent variable, as each unit on the log10-scale means a factor of 10 on metric scale.

$^b$ Results stratified for age and sex.

$^c$ Results stratified for age and sex and adjusted for diabetes, hyperlipidemia, atrial fibrillation, myocardial infarct, coronary artery disease, chronic heart failure, peripheral artery disease, smoking, physical activity, number of teeth and leucocyte count.

$^d$ NA (not available); this parameter would cause collinearity and cannot be calculated.
role of MMP-8 both in local plaque pathology and adverse cardiovascular events is obvious [7,11,12,19–21]. Although we could demonstrate significant differences between stroke subtypes with higher serum values of MMP-8 in stroke due to CE or LVD, these differences are largely explained by CE and LVD contributing to higher stroke severity [22]. However, further research might focus on MMP-8 predicting stroke volume. Using longitudinal study design, impact of MMP-8 predicting ischemic stroke in certain stroke etiologies (e.g. asymptomatic carotid stenosis) as well as the impact on stroke prognosis might be evaluated. This might facilitate decision-making process with regards to therapy adjudication (e.g. carotid endarterectomy), as usage in certain clinical situation is questionable. In addition, utilizing of MMP-8 determination in clinical practice might also potentially help to identify stroke mimics in future, avoiding application of risky therapies such as iv-t-PA.

Circulating TIMP-1 concentrations have been consistently associated with CVD development and prognosis including stroke [7,23,24] and its associations especially with fatal events have been repeatedly shown [7,25–27]. In our recent study including several biomarkers, TIMP-1 concentrations were directly associated with the highest hazards for stroke and death, and including the biomarker to the established risk profile improved the risk discrimination [7]. In addition of being an inhibitor of several MMPs, TIMP-1 plays an important regulatory role in numerous biological pathways, such as proliferation, apoptosis, and angiogenesis [28]. In the present study TIMP-1 did not independently associate with stroke after adjusting for confounders. However, when all measured biomarkers were entered simultaneously into the final model, TIMP was independently associated with stroke and its OR was the highest (9.5/10 units, p < 0.001), describing the importance of the whole proteolytic and oxidative tissue destructive cascade.

In the present study, MPO had a strong association with both stroke and its severity, which is in agreement with earlier studies [4,8]. It has also been reported to predict poor outcome in patients with significant stenosis [29,30] and in CVD-free populations [7,31]. It is a well-known inflammatory marker produced mainly by neutrophils as a response to microbial insult. In addition, MPO enhances the proteolytic activity of MMP-8 and inactivates TIMP-1 and has, therefore, synergetic effect for MMP-8 function [32].

The sources of elevated serum MMP-8 and MPO in CVD has remained partially unclear. MMP-8 and MPO are stored in different neutrophil granules and released by degranulation in response to inflammatory stimuli [33]. Recent studies demonstrated that t-PA treatment in ischemic stroke patients led to neutrophil degranulation with consecutive peaks of MMP-8 and MPO followed by increased production of TIMP-1 [34]. As indicated by sensitivity analyses, tPA treatment did not significantly contribute to higher biomarker concentrations in our study. Overall, the serum concentrations of these biomarkers are higher than those of plasma probably due to the sample preparation, where the anticoagulants inhibit the clotting cascade and complement activation [35]. Therefore, the deviation of serum MMP-8 and MPO concentrations, but not plasma, are partially explained by the genetic variation of the complement factor H [36,37]. However, these SNPs did not significantly associate with the risk of prevalent or incident stroke in a large cohort with a relatively low number of cases [37]. Further research is needed to investigate the usefulness of serum or plasma biomarkers in stroke and the genetic variation of the complement cascade predisposing to stroke. Our study has several limitations. Lower response rate in controls might potentially bias study results. We did not assess infarct volume in stroke patients and there are no data available on C-reactive protein or renal function in controls. Longitudinal study design might have given more information on the course of MMP-8, TIMP-1 and MPO in ischemic stroke. Strengths of our study include the ample information on stroke risk factors, stroke etiology and stroke severity.

4.1. Conclusion

Our data demonstrate significantly higher serum concentrations of inflammatory biomarkers MMP-8, MPO and TIMP-1 in acute ischemic stroke cases compared to controls, even after adjustment for signs of inflammation (leucocyte count). Serum levels of MMP-8 and MPO were strongly associated with stroke severity and stroke etiology.

Conflict of interest

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

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Fig. 1. Distribution of biomarkers by stroke severity and stroke etiology.

(A) Distribution of biomarkers by NIHSS on admission (means and 95% CIs). In this graph, MMP-8/TIMP-1 molar ratio has been multiplied by a factor 1000 for scaling reasons.

(B) Distribution of biomarkers by TOAST classification (means and 95% CIs). In this graph, MMP-8/TIMP-1 molar ratio has been multiplied by a factor 1000 for scaling reasons.
References


