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ORIGINAL ARTICLE

Small Vessel Disease Is Associated with Tissue Inhibitor of Matrix Metalloproteinase-4 After Ischaemic Stroke

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Abstract

Small vessel disease (SVD) is frequent in aging and stroke patients. Inflammation and remodeling of extracellular matrix have been suggested as concurrent mechanisms of SVD. We investigated the relationship between imaging features of SVD and circulating metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) in patients with ischaemic stroke. In patients treated with intravenous thrombolysis, we took blood samples before intravenous thrombolysis and 90 days after the acute stroke and analysed levels of MMPs and TIMPs. We assessed leukoaraiosis, number of lacunes and brain atrophy on pre-treatment CT scan and graded global SVD burden combining such features. We investigated associations between single features, global SVD and MMPs and TIMPs at baseline and at follow-up, retaining univariate statistically significant associations in multivariate linear regression analysis and adjusting for clinical confounders. A total of 255 patients [mean (±SD) = 68.6 (± 12.7) years, 154 (59%) males] were included, 107 (42%) had no signs of SVD; 47 (19%) had from moderate to severe SVD burden. A total of 107 (42%) patients had no signs of SVD; 47 (19%) had from moderate to severe SVD burden. After adjustment, only TIMP-4 proved associations with SVD features. Brain atrophy was associated with baseline TIMP-4 ($\beta = 0.20$;$p = 0.019$) and leukoaraiosis with 90 days TIMP-4 ($\beta = 0.19$; $p = 0.013$). Global SVD score was not associated with baseline TIMP-4 levels ($\beta = 0.10$; $p = 0.072$), whereas was associated with 90 days TIMP-4 levels ($\beta = 0.21$; $p = 0.003$). Total SVD burden was

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associated with higher TIMP-4 levels 90 days after stroke, whereas was not during the acute phase. Our results support a biological relationship between SVD grade and TIMP-4.

Keywords Small vessel disease · Matrix metalloproteinase · Tissue inhibitor of matrix metalloproteinase · Acute stroke

Introduction

Cerebral small vessel disease (SVD) refers to a broad range of pathological processes that affect microcirculation in the brain [1]. SVD becomes more frequent with ageing, is responsible of around one fourth of all strokes and is the main determinant of vascular dementia. The imaging phenotype of SVD is wide and ranges from white matter changes (WMCs) to cerebral microbleeds [2]. Magnetic resonance (MR) is the gold standard imaging tool for both research and clinical investigation of SVD. However, in acute stroke setting, computed tomography (CT) is routinely used for evaluation of candidates for recombinant tissue-plasminogen activator (rt-PA) treatment and may also provide useful information on SVD features.

Although pathogenesis of SVD is poorly understood, a growing body of evidence suggests that chronic inflammation and extra cellular matrix remodelling may predate and exacerbate progression of SVD [3, 4]. A wide range of inflammatory molecules has been studied in relation to SVD in both acute and chronic phase of stroke, with conflicting results [5]. Some authors hypothesized a blood-brain barrier dysfunction as primary pathological process of SVD [6, 7], with a concurrent role of matrix metalloproteinases (MMPs) [3, 8]. MMPs and their tissue inhibitors (TIMPs) are inflammatory molecules involved in breaking down tight junctions and remodeling components of extracellular matrix, contributing to blood-brain barrier damage [9]. Circulating MMPs and TIMPs have been mainly investigated in regard to ischaemic cascade and clinical outcomes on acute stroke [10, 11]; however, current information on SVD and circulating MMPs and TIMPs is lacking.

In a population of ischaemic stroke patients treated with rt-PA, we aimed to evaluate the cross-sectional relationship at two different time points (baseline and 90 days) between single and combined SVD features on plain CT scan and levels of circulating MMPs and TIMPs.

Methods

Patients

We retrospectively analyzed data on eligible participants from the bioloGical MArkers in acute Ischaemic Stroke study (MAGIC). Briefly, patients with acute ischaemic stroke treated with i.v. thrombolysis were prospectively enrolled across 14 centres in Italy. Each patient fulfilled the Safe Implementation of Thrombolysis Stroke-International Stroke Thrombolysis Register (SITS-ISTR) criteria [12]. Study protocol was approved from local ethic committees. Each patient gave written informed consent. Demographic and clinical data were collected. Stroke severity was assessed using the Neurological Institutes of Health Stroke Scale (NIHSS) and administered before rt-PA treatment.

Imaging and Laboratory Procedures

Each patient included in the main study (MAGIC) had computed tomography (CT) scan study at baseline and between 22 and 36 hours after treatment. Each patient had a peripheral blood sample taken to measure levels of circulating biomarkers (MMPs and TIMPs) at two time points: at baseline, during the acute stroke phase before rt-PA administration and 90 days after the index stroke. To minimize variability in assessment, all blood samples were analyzed at a central laboratory based in Florence, Careggi Hospital. Levels of MMPs and TIMPs were determined using Bio-Plex suspension array system (Bio-Rad Laboratories Inc., Hercules, CA, USA) and R&D Kits (R&D System, Milan Italy) following manufacturer's instructions. The coefficient of variation of MMPs and TIMPs assays was 5.8 and 6.8%, respectively.

SVD Assessement

In the present study, we included patients with available baseline or follow-up CT scan. We centrally collected CT scans from participating centres. Three independent trained stroke neurologists (FA, BP, VP) assessed leukoaraiosis, lacunes, and brain atrophy blinded to clinical data, following the STRIVE (STandards for ReportIng Vascular changes on nEuroimaging) recommendations [2]. We performed preliminary intra-class correlation coefficient (ICC) among the three readers on 40 CT scans. Where baseline CT scan was not available, we assessed SVD features on follow-up scan. If the index infarct at the follow-up scan was too large to allow the rating of SVD features, we assessed SVD features only in the contralateral (i.e. non-acutely ischaemic) hemisphere. We graded white matter changes (i.e. leukoaraiosis) with Van Swieten Scale (VSS) in anterior (range 0–2) and posterior (range 0–2) periventricular white matter, then we combined the scores into a five-point ordinal scale [13]. We counted number of lacunes, defined as round or ovoidal shaped hypodense lesions measuring ≤ 20 mm in diameter on axial section in the white matter, basal ganglia or brainstem. We defined brain atrophy as central

and cortical and rated with a three-point ordinal scale as none, moderate or severe against a reference CT brain template [14]. We summed the central and cortical scores to obtain a five-point global cerebral atrophy score (0–4).

We created an aggregate SVD score by summing the scores of white matter changes, lacunes, and brain atrophy. The score has been previously tested in relation to blood-brain barrier permeability [15], white matter perfusion [16], and clinical outcomes [17]. We assigned one point for each of the following if present: severe lucencies (VSS ≥ 2) in anterior or posterior periventricular white matter, lacunes ≥ 2 and severe (≥ 2) brain atrophy. Therefore, the combined four-point ordinal score assessed the global burden of SVD ranging from 0 (no imaging features of severe SVD) to 3 (imaging features of SVD scored as severe for each imaging variable). We divided the study population accordingly.

Statistical Analysis

To obtain normally distributed data, we performed a natural logarithmic transformation of crude values of MMPs and TIMPs. We described demographic and clinical characteristics of the population with summary statistics and used Pearson χ^2, Kruskall-Wallis, or ANOVA test, as appropriate to test differences among groups. To investigate unadjusted associations, we analyzed the distribution of baseline and 90-day levels of MMPs and TIMPs among the SVD features and SVD score using analysis of variance (ANOVA). We used the false discovery rate (FDR) [18] as post hoc test to avoid false positive results. We retained statistically significant associations from the univariate analysis after post hoc analysis and built a multivariable linear regression model adjusting for age, sex, stroke severity, hypertension, diabetes and smoking, with levels of MMPs and TIMPs as dependent variable. We considered statistically significant a p value < 0.05. We carried out statistical analysis with SPSS for Windows (version 22.0; SPSS, Armonk NY, IBM Corp.).

Results

Characteristics of the Study Population

A total of 262 (80%) of the whole MAGIC study population ($n = 327$) had available baseline or follow-up CT scan to assess leukoaraiosis, lacunes and brain atrophy. The main reason for the missed CT scans was transferral problem for central review. Seven patients out of 262 had poor quality of source CT images to reliably assess SVD and were therefore excluded. This left 255 patients at baseline for the final analysis. At 90 days, 21 (8%) patients had died, and 44 (18%) of the remaining patients did not have the follow-up blood sample. This left 190 (75%) patients for the 90-day analysis (Supplemental Fig. 1). There were no differences among demographical, clinical and risk factors between patients with and without blood sample at follow-up (not shown).

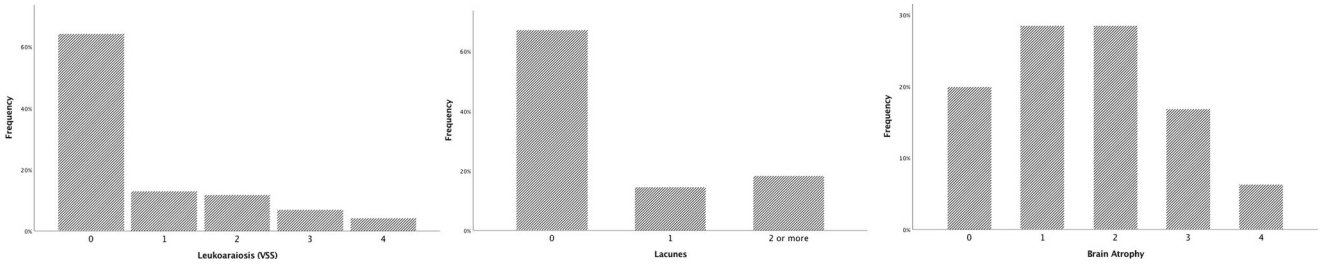
Baseline characteristics of included patients were similar to those of excluded patients except for a longer onset to treatment time (150 vs 165 min, $p = 0.003$) for included patients (Supplemental Table 1). Mean age of the study population was 68.5 ± 12.7 years, 154 (59%) patients were male. Median NIHSS was 12 (IQR = 7–17), median symptoms onset to rt-PA time was 163 min (IQR = 135–180), hypertension was the most frequent risk factor (160 patients, 62%). ICC among the three readers showed a good to very good overall interrater agreement (ICC for leukoaraiosis = 0.87; ICC for lacunes = 0.76; ICC for brain atrophy = 0.89). Distribution of single SVD features is shown in Fig. 1. Combined features into the global SVD score revealed 107 (42%) patients with no signs of SVD, 101 (40%) with mild SVD, 37 (15%) with moderate SVD and 10 (4%) with severe SVD. Patients with increasing SVD grade were older ($p < 0.001$), had higher baseline systolic and diastolic blood pressure values ($p < 0.001$ and $p = 0.003$, respectively) and more frequently history of hypertension ($p < 0.001$) and atrial fibrillation ($p = 0.005$) (Table 1).

Single SVD Features and Levels of MMPs and TIMPs

Univariate analysis of baseline levels of MMPs and TIMPs among single SVD features showed that lacunes were associated with increased levels of MMP-7 ($p = 0.004$), and brain atrophy was associated with TIMP-4 levels ($p < 0.001$) (Supplemental Fig. 2). In multivariate analysis, only brain atrophy showed an independent association with baseline TIMP-4 ($\beta = 0.20$; $p = 0.019$). Analysis of 90-day levels of MMPs and TIMPs showed that leukoaraiosis, number of lacunes and brain atrophy were consistently associated with TIMP-4 levels ($p < 0.001$, $p = 0.001$ and $p < 0.001$, respectively) (Supplemental Fig. 2). Brain atrophy was also associated with MMP-1 ($p = 0.007$) and TIMP-1 ($p = 0.010$), but not after multivariate adjustment. In multivariate analysis, only leukoaraiosis ($\beta = 0.19$; $p = 0.013$) was independently associated with TIMP-4 levels. There were no other statistically significant associations among MMPs, TIMPs and SVD features (not showed).

Global SVD Burden and MMPs and TIMPs

Complete data of the panel of inflammatory markers and univariate associations between all MMPs, TIMPs across SVD score are showed in Table 2 (baseline) and Table 3 (90 days). Baseline levels of MMP-1 (0.85 ng/ml for SVD = 0; 1.01 ng/ml for SVD = 1; 1.6 ng/ml for SVD = 2; 2.2 ng/ml for SVD = 3; $p = 0.006$) and TIMP-4 (0.69 ng/ml for SVD = 0; 0.89 ng/ml for SVD = 1; 1.04 ng/ml for SVD = 2; 1.10 ng/ml for SVD = 3; $p = 0.001$) were associated with SVD score severity at the univariate analysis. At 90-day follow-up, MMP-1 (0.47 ng/ml for SVD = 0; 0.95 ng/ml for SVD = 1; 1.49 ng/ml for SVD = 2;



VSS=Van Swieten Scale.

Fig. 1 Distribution of SVD imaging features in the study population ($N = 255$)

1.90 ng/ml for SVD = 3; $p = 0.010$) and TIMP-4 (0.56 ng/ml for SVD = 0; 0.83 ng/ml for SVD = 1; 1.25 ng/ml for SVD = 2; 1.26 ng/ml for SVD = 3; $p < 0.001$) levels were associated with increasing SVD (Fig. 2). After multivariate adjustment, SVD score was independently associated with TIMP-4 levels at 90 days ($\beta = 0.21$; $p = 0.003$), whereas there was a non-statistically significant trend with baseline TIMP-4 levels ($\beta = 0.10$; $p = 0.072$). SVD score was not independently associated with MMP-1 levels, neither at baseline nor at 90 days.

Discussion

In patients with ischaemic stroke treated with intravenous rt-PA, we investigated SVD features in relation to a large panel of circulating MMPs and TIMPs at baseline and 90 days after the index stroke. We found that brain atrophy was independently related with baseline TIMP-4 levels, and leukoaraiosis was associated with 90 days TIMP-4. Global SVD burden, expressed by combining single SVD features, was associated with TIMP-4 levels at 90 days, with a trend at baseline. SVD was not associated with any of the remaining MMPs and TIMPs.

SVD acts as a composite product of ageing, vascular risk factors and several different pathologies, resulting in three main imaging features detectable with CT scan (leukoaraiosis, lacunes and atrophy). Recently, the concept of a comprehensive assessment of SVD has been validated in relation to cognitive outcomes [19] and cardiovascular risk factors [20], and in acute stroke setting, SVD burden has been associated with poorer clinical outcomes [17, 21]. Diverse mechanisms, such as concurrent presence of inflammatory and tissue remodeling activity, may play a role on development of SVD [22].

Table 1 Baseline characteristics of study population

	Total $N = 255$	SVD score = 0 $N = 107$	SVD score = 1 $N = 101$	SVD score = 2 $N = 37$	SVD score = 3 $N = 10$	p
Age, years (±SD)	68.6 ± 12.7	60.9 ± 13.6	72.7 ± 9.0	76.9 ± 4.3	77.3 ± 8.5	< 0.001
Sex, male	154 (59)	57 (53)	64 (63)	22 (60)	8 (80)	0.253
Weight, mean (±SD)	73.5 ± 13.2	73.3 ± 14.4	73.8 ± 12.4	72.9 ± 13.2	74.5 ± 12.3	0.975
Baseline NIHSS, median (IQR)	12 (7–17)	12 (7–17)	12 (7–17)	13 (9–20)	11 (8–13)	0.502
Systolic BP, mmHg, mean (±SD)	147.1 ± 21.7	143.2 ± 22.3	146.7 ± 20.8	157.8 ± 20.2	155.5 ± 19.9	0.003
Diastolic BP, mmHg, mean (±SD)	79.6 ± 12.7	79.2 ± 13.1	77.6 ± 12.3	84.3 ± 10.4	83.0 ± 12.5	0.040
White blood cells, mean (±SD)	7.9 ± 2.5	7.8 ± 2.6	8.2 ± 2.5	8.2 ± 2.8	7.1 ± 0.9	0.424
Glucose, mg/l, mean (±SD)	129.7 ± 49.9	124.9 ± 50.6	136.4 ± 56.0	128.1 ± 30.7	111.0 ± 23.9	0.242
Hypertension	161 (62)	49 (48)	70 (70)	29 (78)	8 (80)	< 0.001
Diabetes	38 (15)	17 (16)	13 (13)	5 (14)	1 (10)	0.893
Dyslipidemia	65 (25)	29 (28)	24 (24)	9 (25)	–	0.288
Atrial fibrillation	58 (23)	14 (13)	27 (27)	14 (39)	1 (10)	0.005
Smoking	72 (33)	22 (24)	30 (35)	15 (48)	2 (33)	0.080
Heart failure	33 (13)	10 (10)	15 (15)	7 (19)	–	0.919
Onset-to-treatment time, median (IQR)	165 (140–180)	165 (140–180)	165 (130–190)	167 (150–186)	183 (170–205)	0.149

Data are numbers (%) unless otherwise stated. SVD = Small Vessel Disease; VSS=Van Swieten Scale; SD = Standard Deviation; NIHSS=National Institutes of Health Stroke Scale; BP=Blood Pressure; IQR = Interquartile Range

Table 2 Baseline levels (mean ± standard deviation) of matrix metalloproteinases and their tissue inhibitors across small vessel disease score

	SVD score = 0 *N* = 107	SVD score = 1 *N* = 101	SVD score = 2 *N* = 37	SVD score = 3 *N* = 10	p
MMP-1 baseline, ng/ml	0.85 (± 1.53)	1.01 (± 1.51)	1.60 (± 1.60)	2.23 (± 1.25)	*0.006**
MMP-2 baseline, ng/ml	5.36 (± 0.62)	5.36 (± 0.65)	5.37 (± 0.67)	5.24 (± 0.36)	0.946
MMP-3 baseline, ng/ml	2.70 (± 0.77)	2.74 (± 0.72)	2.75 (± 0.85)	2.99 (± 0.68)	0.388
MMP-7 baseline, ng/ml	0.22 (± 1.47)	0.35 (± 1.13)	0.71 (± 1.39)	0.70 (± 0.36)	0.100
MMP-8 baseline, ng/ml	2.99 (± 0.86)	2.78 (± 1.02)	3.18 (± 1.03)	3.03 (± 1.07)	0.216
MMP-9 baseline, ng/ml	5.86 (± 0.76)	5.67 (± 0.73)	5.82 (± 0.77)	5.77 (± 1.06)	0.787
TIMP-1 baseline, ng/ml	4.99 (± 0.54)	5.13 (± 0.50)	5.07 (± 0.60)	5.24 (± 0.38)	0.427
TIMP-2 baseline, ng/ml	4.58 (± 0.31)	4.60 (± 0.23)	4.62 (± 0.28)	4.63 (± 0.10)	0.954
TIMP-4 baseline, ng/ml	0.69 (± 0.55)	0.89 (± 0.52)	0.98 (0.57)	1.10 (± 0.44)	*0.001**

All values are log-transformed. *SVD* small vessel disease, *MMP* matrix metalloproteinase, *TIMP* tissue inhibitor of metalloproteinase

*Statistically significant after false discovery rate

Histological studies demonstrated presence of inflammatory cells (i.e. macrophages) [23] and increased microglial activation around white matter changes [24]. Increased levels of MMPs, as expression of inflammation and tissue remodeling activity, have been found in patients with white matter changes and in cerebrospinal fluid of patients with vascular cognitive impairment [8, 25]. Nonetheless, previous studies on circulating biomarkers and SVD evaluated a broad range of molecules and failed to identify a blood biomarker for SVD, reporting contradictory results [5, 26–28]. We found a potentially novel association between SVD features, such as brain atrophy and leukoaraiosis, and TIMP-4 90 days after acute stroke. Although brain atrophy could express both a neurodegenerative and concurrent SVD process, leukoaraiosis has been historically identified as a specific marker of SVD. Furthermore, at 90 days, there was also a meaningful association between increasing severity of SVD and TIMP-4 levels with a dose-response effect, suggesting a biological gradient and reinforcing the relationship. Although we acknowledge that the SVD score needs validation in different cohorts and with larger sample size, we advocate that the assessment of global SVD burden may convey more information than single SVD feature evaluation.

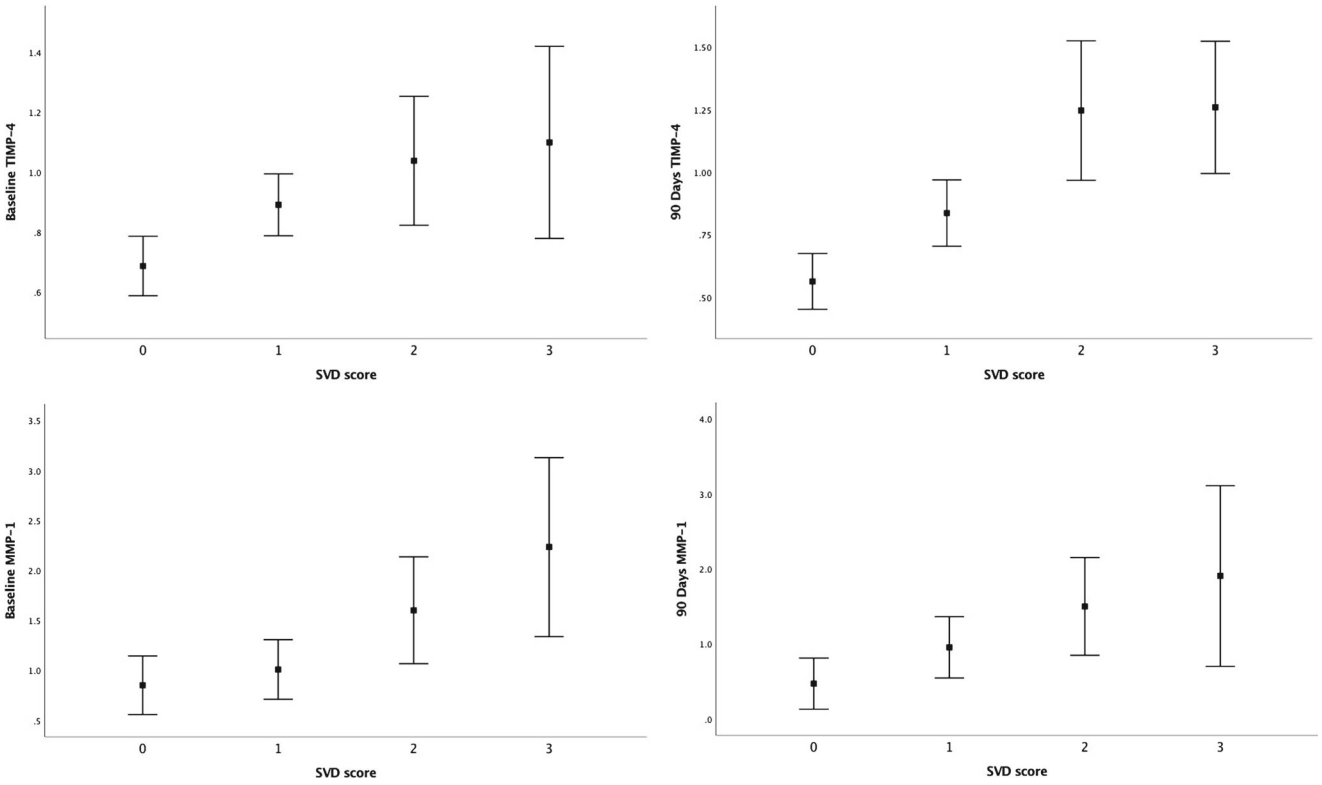
TIMP-4 has been found in brain, heart and skeletal muscles and inhibits a wide range of MMPs, exerting protective effects on remodeling of extracellular matrix [29]. Our results showed a similar increase of circulating levels of TIMP-4 in patients with the increasing grade of SVD burden. A possible link between TIMP-4 and SVD is the regulatory effect exerted by TIMP-4 on smooth muscle cells. In experimental model of hypertensive mice, TIMP-4 seems to protect smooth muscle cells from migration and contrasting the vessel remodeling [30]. Smooth muscle cells are typically reduced in SVD through uncertain mechanisms [1], and we may speculate that TIMP-4 increases to contrast the progression of SVD, eventually targeting

Table 3 Ninety-day levels (mean ± standard deviation) of matrix metalloproteinases and their tissue inhibitors across small vessel disease score

	SVD score = 0 *N* = 107	SVD score = 1 *N* = 101	SVD score = 2 *N* = 37	SVD score = 3 *N* = 10	*p*
MMP-1, 90 days, ng/ml	0.46 (± 1.58)	0.95 (± 1.83)	1.50 (± 1.61)	1.90 (± 1.44)	*0.010**
MMP-2, 90 days, ng/ml	5.48 (± 0.60)	5.55 (± 0.95)	5.58 (± 0.70)	5.50 (± 0.49)	0.917
MMP-3, 90 days, ng/ml	2.65 (± 0.72)	2.94 (± 0.83)	2.81 (± 0.95)	3.20 (± 0.56)	0.064
MMP-7, 90 days, ng/ml	0.24 (± 1.23)	0.67 (± 1.32)	0.68 (± 1.57)	0.64 (± 1.35)	0.144
MMP-8, 90 days, ng/ml	2.60 (± 1.00)	2.55 (± 0.99)	2.43 (± 0.86)	2.73 (± 1.05)	0.852
MMP-9, 90 days, ng/ml	5.17 (± 0.71)	5.33 (± 0.82)	4.98 (± 0.64)	5.31 (± 0.66)	0.177
TIMP-1, 90 days, ng/ml	5.06 (± 0.45)	5.23 (± 0.42)	5.24 (± 0.71)	5.51 (± 0.43)	0.021
TIMP-2, 90 days, ng/ml	4.60 (± 0.36)	4.65 (± 0.27)	4.70 (± 0.25)	4.67 (± 0.19)	0.482
TIMP-4, 90 days, ng/ml	0.56 (± 0.50)	0.84 (± 0.59)	1.25 (± 0.69)	1.26 (± 0.32)	*<0.001**

All values are log-transformed. *SVD* small vessel disease, *MMP* matrix metalloproteinase, *TIMP* tissue inhibitor of metalloproteinase

*Statistically significant after false discovery rate



SVD=Small Vessel Disease; TIMP=Tissue Inhibitor of Matrix Metalloproteinase; MMP=Matrix Metalloproteinase.

Fig. 2 Univariate associations between SVD score and inflammatory markers (only statistically significant markers after false discovery rate are showed). Data are means (95% confidence intervals)

smooth muscle cells. Furthermore, a role of TIMP-4 on collagen- and thrombin-induced platelet inhibition has been reported [31]. SVD has been associated with increased platelet activation [32] and pro-coagulative state [33]. Therefore, in patients with cerebral SVD, TIMP-4 might have pleiotropic biological effects, ranging from regulation of remodeling of extracellular matrix and smooth muscle cell growth to a key molecule in mitigating blood coagulation and particularly platelet aggregation.

Levels of MMPs and TIMPs may rise in the first hours of stroke, confounding associations with SVD, and this could explain why we failed to demonstrate consistent associations at baseline, except brain atrophy. It is plausible that evaluation of circulating biomarkers related to SVD may be informative at the steady-state level rather than at the acute phase level. Again, although MMP-9 and TIMP-1 seem to be involved in early hours after stroke [10, 11], our results suggest a different molecule profile implicated in SVD, possibly reflecting the chronic nature of the disease (SVD) rather than the acute phase of the stroke. Hence, the association we found at 90 days (i.e. steady state) between SVD and TIMP-4 is novel and biologically plausible.

Our study has limitations. First, we investigated SVD features in relation to a large panel of MMPs and TIMPs, and one could argue that our findings reflect a statistical fishing expedition approach. However, we used a post hoc analysis (false discovery rate) to limit the number of false positive results. Second, we did not use the gold standard for diagnosis of SVD (i.e. MR), but there is substantial agreement between CT and MR in rating of white matter changes and atrophy [34]. We rated only preexisting lacunar infarcts detectable on plain CT scan (i.e. cavitated lacunar lesions), which represent around 20% of all small subcortical infarcts detectable with MR scan [35]. However, with the limitation of CT scan, we found a rate of SVD hallmarks comparable to previous studies [18, 36]. Furthermore, availability of CT scans for central reading was 80% of the whole study population. Nonetheless, comparison between baseline characteristics of included and excluded patients did not support this hypothesis, showing a comparable risk factor profile and therefore similar SVD occurrence. Finally, the retrospective nature of the study and the lack of a control group (i.e. patients not treated with rt-PA) did not allow any causal inference between SVD and TIMP-4; however, our results could be further explored in future studies with appropriate design. Strengths of our study were the large

sample size in relation to SVD, MMPs and TIMPs in patients with stroke, the use of validated and reproducible scales for SVD assessment, the blinded CT rating of SVD features by three independent reviewers and the measurements of circulating biomarkers at two different time points.

In conclusion, in a population of ischaemic stroke patients, we showed that brain atrophy was associated with baseline TIMP-4 levels and leukoaraiosis was associated with 90-day TIMP-4 levels. A global SVD score, expressed as a combined product of leukoaraiosis, lacunes and brain atrophy, was associated with TIMP-4 levels at 90 days with a dose-response effect, supporting the concept of evaluation of total SVD burden. Our results showed that increasing grade of SVD sustains higher levels of TIMP-4 and supports the involvement of TIMP-4 in the pathologic process of SVD. Further studies to investigate whether TIMP-4 may serve as a circulating biomarker of development and progression of SVD are warranted.

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Compliance with Ethical Standards

Study protocol was approved from local ethic committees. Each patient gave written informed consent.

Conflict of Interest The authors declare that they have no conflict of interest.

Ethical Approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study.

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