

Matrix Metalloproteinases Are Associated With Increased Blood–Brain Barrier Opening in Vascular Cognitive Impairment

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Background and Purpose—Subcortical ischemic vascular disease (SIVD) is a major form of vascular cognitive impairment (VCI) due to small vessel disease. Matrix metalloproteinases (MMPs) are neutral proteases that disrupt the blood–brain barrier and degrade myelin basic protein under conditions of neuroinflammation. Brain tissues and cerebrospinal fluid (CSF) of patients with VCI have increased levels of MMPs. We hypothesized that patients with SIVD have increased MMPs in the CSF, which are associated with increased CSF albumin.

Methods—We studied 60 patients with suspected VCI. Twenty-five were classified as SIVD, whereas other groups included mixed Alzheimer disease and VCI, multiple strokes, and leukoaraiosis when white matter lesions were present and the diagnosis of VCI was uncertain. MMP-2 and MMP-9 in CSF and plasma were measured by gel zymography and indexed to CSF and plasma albumin. MMP-3 activity was measured by fluorescent assay.

Results—We found reduced MMP-2 index ($P < 0.001$) in the CSF for the full group of patients (SIVD, multiple strokes, mixed Alzheimer disease and VCI, and leukoaraiosis) compared with control subjects, whose CSF was obtained during spinal anesthesia. MMP-3 activity was increased in VCI compared with control subjects ($P < 0.01$). In SIVD, MMP-2 index showed a negative correlation with albumin index, which was absent with the MMP-9 index. Combining MMP-2 index and MMP-3 activity separated the patients with SIVD from the control subjects with high specificity ($P < 0.0005$).

Conclusions—Our results support the hypothesis that MMPs are associated with increased CSF albumin and suggest that they may contribute to the pathophysiology of SIVD. (*Stroke*. 2011;42:1345-1350.)

Key Words: Binswanger disease ■ blood–brain barrier ■ brain ■ matrix proteins ■ pathobiology ■ vascular cognitive impairment ■ vascular dementia ■ white matter disease

Subcortical ischemic vascular disease (SIVD), which is the small vessel form of vascular cognitive impairment (VCI), is a major cause of dementia in the elderly.¹ The characteristic features of SIVD are focal neurological findings, gait imbalance, neuropsychological dysfunction, and large white matter hyperintensities (WMHs) on MRI.² Arteriosclerosis of the small vessels in the deep white matter with demyelination and lacunar strokes are the pathological hallmarks.³ As opposed to the large-vessel form of VCI, the onset is usually insidious and challenging to separate from other forms of neurodegeneration such as Alzheimer disease.⁴ Many investigators are searching for biomarkers that can aid in the early diagnosis of SIVD because it is considered to be the optimal form of VCI for treatment trials.⁵ There is growing evidence from other investigators and us that there is disruption of the blood–brain barrier (BBB) in the white

matter.^{6,7} We have proposed that the BBB damage is due to the induction of matrix metalloproteinases (MMPs) by hypoxic/ischemic injury.⁸ Normally, MMPs are present in the brain in latent forms that are activated to remodel the extracellular matrix.⁹ However, when they are induced and activated under conditions of hypoxia, they can disrupt the basal lamina and tight junctions of the cerebral blood vessels¹⁰ and degrade myelin basic protein.¹¹

Autopsy studies have demonstrated expression of gelatinase A (MMP-2), gelatinase B (MMP-9), and stromelysin-1 (MMP-3) in brains of patients with white matter lesions from stroke, multiple sclerosis, and vascular dementia.^{12–15} In an earlier study of MMPs in cerebrospinal fluid (CSF) in patients with VCI, we observed an increase in MMP-9.¹⁶ However, the MMPs were only measured in the CSF and some of the MMP-9 in the CSF may have come from the

Received August 24, 2010; final revision received November 24, 2010; accepted December 8, 2010.

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The online-only Data Supplement is available at <http://stroke.ahajournals.org/cgi/content/full/STROKEAHA.110.600825/DC1>.

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Stroke is available at <http://stroke.ahajournals.org>

DOI: 10.1161/STROKEAHA.110.600825

blood, particularly if the BBB was disrupted. Using albumin, which is produced in the liver, as an index marker for BBB permeability, it is possible to separate endogenous from exogenous MMP production. By indexing the MMPs in the brain and blood compartments to albumin in both, in a manner similar to the use of the IgG index in multiple sclerosis, it is possible to determine intrathecal synthesis of the enzymes.¹⁷ Therefore, we determined an MMP index for MMP-2 and MMP-9 by measuring the levels in blood and CSF and forming the MMP index. We hypothesized that MMPs were produced in the CSF compartment in patients with SIVD, but not in the patients with other forms of VCI. Furthermore, we used a newly developed fluorescent immunocapture assay to measure the levels of active MMP-3.

Subjects and Methods

Subjects

Sixty patients with suspected VCI were entered into the study. These patients were part of a larger study of multiple parameters to select the optimal biomarkers for SIVD. This report describes the results of the CSF biomarker studies in a subgroup of 45 patients who had a lumbar puncture as part of the evaluation. Patients were referred to 1 of the study neurologists (J.C.A., E.E., G.A.R.) for evaluation and were seen in the Neurology Clinics at the University of New Mexico Hospital and the Albuquerque Veterans Administration Hospital. After obtaining informed consent, they were enrolled in the study. All aspects of the study were conducted in compliance with the regulations of the University of New Mexico Human Research Review Committee and the Albuquerque Veterans Hospital Research Committee. Patients had neurological and neuropsychological testing, lumbar puncture for collection of CSF, and MRI. Imaging studies always preceded lumbar puncture. Study procedures were completed within 2 contiguous days.

CSF was analyzed for routine studies (cells, protein, glucose, culture) and blood was collected for calculation of a demyelinating profile and albumin index (=albumin in CSF/albumin in plasma). CSF was collected from 20 control subjects who had spinal anesthesia for non-neurological conditions.

Diagnoses

Patients entered into the study were referred because of WMHs on MRI, cognitive complaints, and suspected VCI. One patient had dementia, but the others were suspected to have VCI-no dementia.⁴ We separated patients clinically into several diagnostic subgroups of VCI based on the results of the neurological, neuropsychological, and anatomic MRI findings without knowledge of CSF results (Supplemental Table 1; <http://stroke.ahajournals.org>). Clinical categories included: (1) SIVD when small-vessel disease was suspected in the presence of focal findings and significant WMHs on MRI; (2) lacunar or large-vessel infarcts when they were evident on the MRI and accompanied by hemiparesis; (3) mixed vascular and Alzheimer disease for those with symmetrical periventricular WMHs and dominant memory impairment; and (4) a group of patients with leukoaraiosis whose diagnosis was uncertain.¹ Although patients had diagnoses made by 2 neurologists with expertise in VCI (J.C.A. and G.A.R.), they remain provisional without autopsy verification. Only 2 patients have died and undergone autopsy, which confirmed the diagnosis of multiple infarcts in 1 and mixed vascular and Alzheimer disease in another.

All patients had medical evaluations to exclude other causes of white matter disease and cognitive impairment. In all patients with CSF studies, the demyelinating profile revealed no oligoclonal bands, reducing the likelihood of including patients with multiple sclerosis. Volume measurements of the WMHs were made by manually tracing the white matter lesions on fluid-attenuated inversion recovery MRI and summing the volumes from each slice.

Gelatin-Substrate Zymography of CSF and Plasma

CSF and plasma were collected at the same time. The levels of MMP-2 and MMP-9 were measured in the CSF and plasma by gelatin-substrate zymography as described previously.¹⁸ CSF samples (10 μ L) were mixed with an equal volume of loading buffer (62.5 mmol/L Tris-HCl pH 6.8, 10% glycerol, 2% sodium dodecyl sulfate, and 0.00125% bromophenol blue). Plasma samples were diluted 1:30 with distilled water and 10 μ L of the diluted sample was mixed with 10 μ L of loading buffer and applied to the zymography gels. After electrophoresis, gels were washed in 2.5% Triton X-100 to remove the sodium dodecyl sulfate and then incubated for 96 hours at 37°C in a developing buffer containing 50 mmol/L Tris, pH 7.6, 5 mmol/L CaCl₂, 0.2 mmol/L NaCl, and 0.02% Brij-35. Gels were stained with 0.125% Coomassie Brilliant Blue R-250 (Sigma, St Louis, MO) for 30 minutes in 10% acetic acid and 50% methanol. Gels were destained with a solution containing 10% acetic acid until clear bands of gelatinolysis appeared on a dark blue background. Dried gels were scanned and analyzed using AlphaEase software (Alpha Innotech, San Leandro, CA). Media from HT1080 fibrosarcoma cells served as positive controls for human MMP-2 and MMP-9.

Immunocapture Assay and Fluorometric Measurement of MMP-3 Enzymatic Activity

The activity of MMP-3 (stromelysin-1) in CSF and plasma was measured fluorometrically using a 5-FAM/QXL520 fluorescence resonance energy transfer peptide (60580; AnaSpec, San Jose, CA). In the intact fluorescence resonance energy transfer peptide (5-FAM-Arg-Pro-Lys-Pro-Val-Glu-Nva-Trp-Arg-Lys[QXL520])-NH₂, the fluorescence of 5-FAM (5-carboxyfluorescein) is quenched by QXL520. On cleavage into 2 separate fragments by MMP-3, the fluorescence of 5-FAM is recovered and can be monitored at excitation/emission wavelengths of 490/520 nm. This peptide has been documented to be cleaved by only MMP-3 and MMP-12, but not by other MMPs.¹⁹

Before conducting the activity assay, the MMP-3 present in the sample was immunocaptured using a rabbit anti-MMP-3 antibody (sc-6839-R; Santa Cruz Biotechnology) to ensure the specificity of the assay for MMP-3. Samples of CSF (50 μ L) and plasma (20 μ L) were mixed in 1 mL of TCNB buffer (50 mmol/L Tris, 10 mmol/L CaCl₂, 150 mmol/L NaCl, 0.05% Brij-35) with 2 μ g of the immunocapture antibody at 4°C for 2 hours in a rocker. Twenty microliters of Protein A/G beads (sc-2003; Santa Cruz Biotechnology) were then added to the tubes and samples were incubated overnight at 4°C with gentle rocking. Samples were centrifuged at 1000 g for 5 minutes at 4°C and supernatant was discarded. Beads were washed twice with 500 μ L of TCNB buffer. After the last wash, 100 μ L of assay buffer (50 mmol/L Tris-HCl, pH 7.6, 200 mmol/L NaCl, 5 mmol/L CaCl₂, 20 μ mol/L ZnSO₄, and 0.05% Brij-35) was added and mixed thoroughly with the beads. Samples were transferred to a black 96-well plate and 100 μ L of assay buffer containing 2 μ mol/L of the fluorescence resonance energy transfer peptide was added. Fluorescence was measured after 8 hours of incubation at 37°C and expressed as relative fluorescence units after subtraction of the readings for the substrate control (without sample) from the relative fluorescence unit values of the samples.

Statistical Methods

Statistical analyses for between-group differences for neuropsychological tests and clinical history were done using SPSS (SPSS for Windows, 16.0.1). One-way analyses of variance and χ^2 analyses were conducted as appropriate based on continuous or categorical data. For the BBB and albumin index data, we determined statistical significance with nonparametric *t* tests or 1-way analysis of variance (Kruskal-Wallis with Dunn corrections for multiple comparisons) and with Spearman rank nonparametric correlations or linear correlations using Prism 5 for Mac (GraphPad, La Jolla, CA). Statistical significance was set at $P < 0.05$. The data were represented as mean \pm SEM.

Table 1. Clinical Information on the Patients in the Groups Identified by Diagnosis (See Text)

	SIVD	MI	MX	LA	Statistics
Male sex (%)	9 (48%)	6 (67%)	6 (100%)	2 (25%)	$\chi^2 (3)=8.75, P=0.03$
Hypertension	10 (53%)	5 (56%)	4 (67%)	0 (0%)	$\chi^2 (3)=8.53, P=0.04$
Diabetes	5 (26%)	1 (11%)	2 (33%)	1 (13%)	$\chi^2 (3)=1.72, NS$
Hyperreflexia	16 (84%)	8 (89%)	3 (50%)	5 (63%)	$\chi^2 (3)=4.57, NS$
Imbalance	18 (95%)	7 (78%)	4 (67%)	3 (38%)	$\chi^2 (3)=10.52, P=0.02$
Stroke history	9 (47%)	9 (100%)	1 (17%)	1 (13%)	$\chi^2 (3)=16.16, P<0.01$
Lesion Size	0.12 (0.13)	0.05 (0.08)	0.06 (0.04)	0.02 (0.03)	$F (3, 37)=2.63, P=0.07$

SIVD indicates subcortical ischemic vascular disease; MI, multiple strokes; MX, mixed Alzheimer disease and vascular cognitive impairment; LA, leukoaraiosis; NS, nonsignificant.

Results

The numbers of patients in each subgroup is shown in Supplemental Table I. Nineteen patients were classified as SIVD with smaller numbers in the other groups. Prevalence of hypertension was significantly increased in all groups except leukoaraiosis; hyperreflexia and stroke were highest in the multiple infarcts group; and patients with SIVD had the largest volume of WMHs (Table 1). Executive function was lowest in the SIVD group and memory function worse in the mixed vascular and Alzheimer disease group but neither was statistically significant (Table 2). Clinical information on the 20 control subjects is shown in Supplemental Table II.

A representative MRI of a patient with SIVD shows extensive white matter changes with lacunar strokes (Figure 1A–C). Symmetrical periventricular lesions are seen in a patient with mixed vascular and Alzheimer disease (Figure 1D–F). MRI in a patient with multiple infarcts shows a large stroke with white matter changes (Figure 1G–I). A patient in the leukoaraiosis group shows scattered WMHs but an uncertain diagnosis (Figure 1J–L). However, the MRI was not diagnostic, particularly in the mixed vascular and Alzheimer disease and leukoaraiosis groups.

To answer the first question of the relationship of the MMP indices to albumin index, we compared all patients in the VCI group, including those with leukoaraiosis that had both measurements completed. The MMP-2 index was significantly lower when the full group was compared with the control subjects (Figure 2A). Similarly, MMP-3 activity was higher in VCI than in control subjects (Figure 2B). There was a negative correlation between the MMP-2 index and the albumin index (Figure 2C), but not in the MMP-9 index (Figure 2D).

Analyzing the MMP-2 index for the different diagnostic groups, we found a significant difference between the control subjects and the patients with SIVD ($P<0.05$; Figure 3A). Comparing the SIVD group with albumin index improved the separation between patients and control subjects and showed a negative correlation between the MMP-2 index and albumin index for the patients with SIVD ($P<0.04$; Figure 3B).

Because we hypothesized that MMPs would be associated with WMHs in the SIVD group, we plotted MMP-2 index against MMP-3 activity for the SIVD and control groups. A scatterplot showed the clustering in different quadrants of the SIVD and control values (Figure 4A). Control subjects had a high MMP-2 index and low MMP-3 activity placing them in 1 quadrant of the graph (upper left). On the other hand, patients with SIVD had a low MMP-2 index and high MMP-3 activity and were mainly clustered in another quadrant (lower right). To determine the statistical significance of the clusters, we formed the ratio of MMP-2 index to MMP-3 activity for the SIVD and control subjects. The histogram of MMP-2 index/MMP-3 activity for control subjects and patients with SIVD shows that these 2 groups could be separated with the mean of control subjects located at 0.00234 and mean of SIVD located at 0.01232 ($P<0.0005$; Figure 4B).

Discussion

We found a reduction in the MMP-2 index and an increase in MMP-3 activity in CSF of patients with VCI. Albumin index, which is a marker of BBB damage, showed a significant correlation with the MMP-2 index, suggesting an association between the reduction in MMP-2 and opening of the BBB. We identified an active form of MMP-3 in CSF using a sensitive immunocapture method for the first time in CSF.

Table 2. Neuropsychological Test Results for Patients in the Groups Identified by Diagnosis

	SIVD	MI	MX	LA	Statistics
MMSE*	27.28 (2.76)	28.12 (2.03)	24.40 (4.45)	27.38 (2.50)	$F (3, 35)=1.91, NS$
Executive function†	41.29 (7.49)	43.00 (9.84)	41.33 (6.02)	44.88 (8.32)	$F (3, 35)=0.41, NS$
Memory†	41.82 (13.62)	38.75 (11.67)	34.33 (8.17)	46.12 (8.22)	$F (3, 35)=1.31, NS$
Language†	43.88 (9.64)	42.12 (4.67)	37.17 (8.33)	49.38 (10.17)	$F (3, 35)=2.30, NS$
Attention†	41.82 (7.34)	42.88 (5.41)	40.00 (6.96)	42.62 (4.34)	$F (3, 43)=0.27, NS$

SIVD indicates subcortical ischemic vascular disease; MI, multiple strokes; MX, mixed Alzheimer disease and vascular cognitive impairment; LA, leukoaraiosis; MMSE, Mini-Mental State Examination; NS, nonsignificant.

*Mean (SD).

†Standardized T-scores.

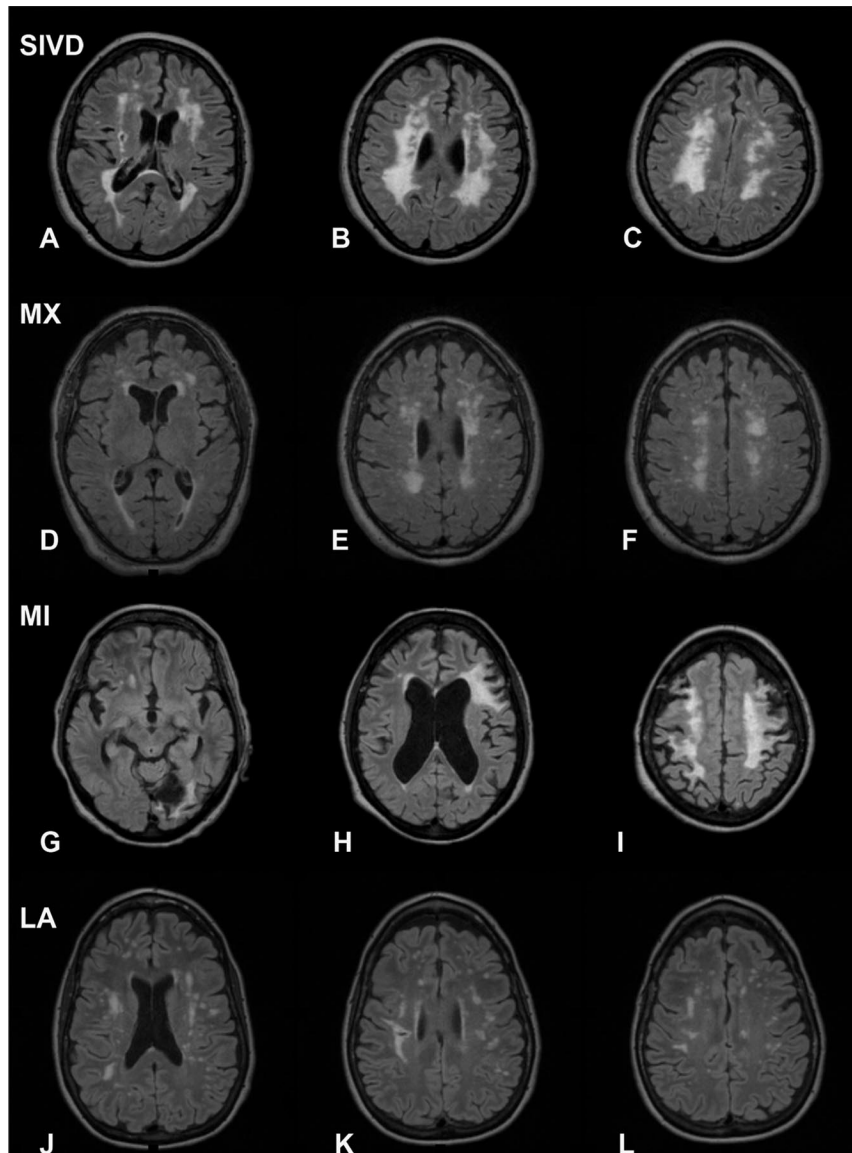


Figure 1. Fluid-attenuated inversion recovery MRI scans from representative patients in the different subgroups. **A–C**, Patients in the subcortical ischemic vascular disease (SIVD) group show extensive white matter hyperintensities (WMHs) in a relatively symmetrical distribution. **D–F**, Mixed VCI and Alzheimer disease (MX) patients have WMHs that are also symmetrical. **G–I**, Patients with multiple infarcts (MI) have asymmetrical lesions consistent with strokes. **J–L**, Patients with leukoariosis (LA) have different patterns of WMHs that are difficult to characterize. VCI indicates vascular cognitive impairment.

The ratio of MMP-2 index and MMP-3 activity separated the SIVD group from the control subjects. Our results support the hypothesis that MMPs are associated with white matter damage, particularly in SIVD.

Pathological studies in patients with SIVD often show loss of myelin and small-vessel infarcts that are associated with fibrosis of blood vessels secondary to arteriolosclerosis. Tissue hypoxia is suggested to occur secondary to damaged blood vessels with hypoxic hypoperfusion.^{20–22} In SIVD, demyelination is often seen around vessels with evidence of leakage across a disrupted BBB.^{23–25} Hypoxic conditions initiate an inflammatory reaction with the expression of MMPs, which could both contribute to the opening of the BBB and breakdown of myelin.

In our earlier study measuring MMPs in CSF of patients with VCI, we found MMP-9 was elevated.¹⁶ However, in this study, MMP-9 index failed to show a significant increase over the control subjects. In the earlier study, which was done with CSF measurements alone, the contribution to the CSF of MMPs in the blood was unknown. Because proteins mea-

sured in the CSF could be transferred from the blood into the CSF through a damaged BBB, which is known to be present in VCI, the use of an index is more accurate.¹⁷ Another explanation for the differences between the 2 studies could be the more advanced disease in the earlier series with many of the patients in the vascular dementia category, whereas only 1 in the current series had that designation.

We found that the MMP-2 index, which was calculated similarly to the IgG index in multiple sclerosis, was lower in the patients with VCI as a whole compared with control subjects and was lowest in the SIVD subgroup. A reduction in MMP-2 was not observed in the CSF in the earlier study. This could have been because the amount of MMP-2 in the CSF is much larger than MMP-9, and small changes could have been missed.

The measurement of MMP-2 in the CSF and plasma was done by zymography, which detects the 72-kDa latent form of the enzyme. There are 2 possible explanations that could explain the decrease in the latent form of the enzyme. On the 1 hand, activation of MMP-2 could deplete the latent form.

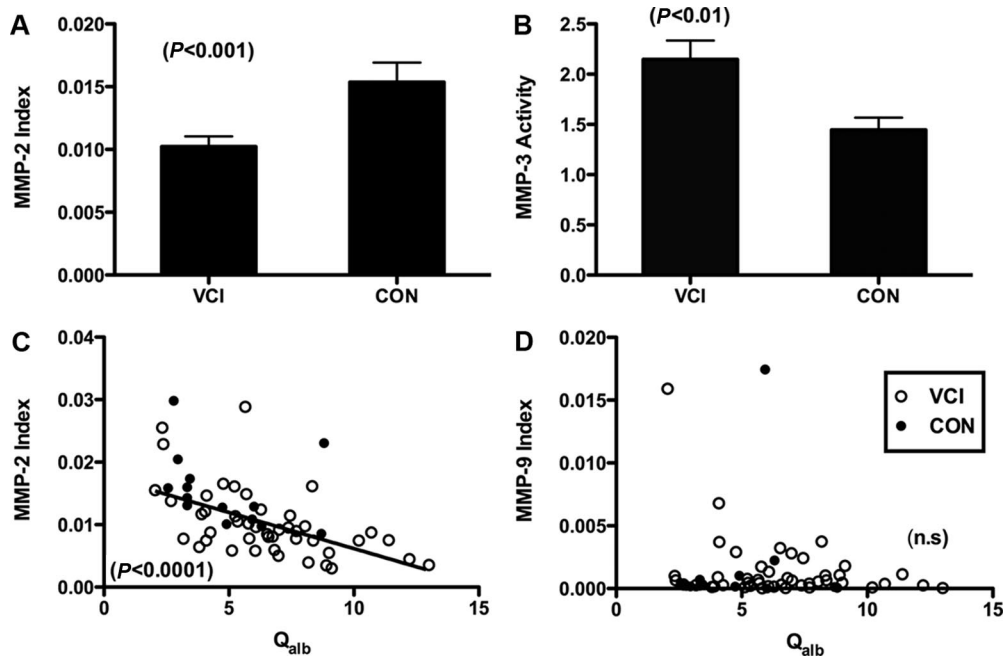


Figure 2. Cerebrospinal fluid values for the matrix metalloproteinase-2 (MMP-2) index and MMP-3 activity. **A**, MMP-2 index for VCI and control subjects. The MMP-2 index for the VCI group was significantly lower than controls. **B**, MMP-3 activity is increased compared with control subjects ($P < 0.01$). Significance levels are shown in parentheses. **C**, MMP-2 index values for all patients with VCI (open circles) and control subjects (black dots) are plotted against albumin index (Q_{alb}). There was a significant negative correlation ($P < 0.0001$). **D**, MMP-9 index plotted similarly failed to show a correlation. VCI indicates vascular cognitive impairment.

Another possibility is that the astrocytes may be damaged or dying, resulting in a reduction in the production of the latent form. Resolution of this issue is not possible at this time.

Neither MMP-2 index nor MMP-3 activity clearly separates patients and control subjects, suggesting that these are not biomarkers themselves. However, the relationship between MMP-2 index and the albumin index shows an association between the levels of MMPs and the disruption of the BBB. We propose that elevated MMPs could contribute both to BBB opening and demyelination. The main biomarker for SIVD appears to be the MMP-2 index, which was consistently lower in SIVD compared with the other groups and control subjects. Astrocytes are a major source of MMP-2, and white matter is gliotic in SIVD. The MMP-3 activity was more variable. Macrophages and pericytes express MMP-3 around blood vessels in regions of demyelination in VCI brains, but the source of MMP-3 is uncertain.¹⁵

The MRI scans aided along with clinical features in identifying the patients with SIVD. SIVD produces a pattern of white matter damage in some patients that suggests gradual growth of existing lesions.²⁶ It is unlikely that such a pattern

is the result of a series of silent strokes that occur in both hemispheres in a relatively symmetrical fashion with sparing of the U-association fibers. We propose that the MMPs participate in an ongoing inflammatory reaction with gliosis and macrophage recruitment, responding to hypoxia and blood vessel fibrosis. Although the data support a link between MMPs and BBB disruption, the overlap of the data from the patients and control subjects precludes their use alone as a biomarker. We propose that hypoxia to deep white matter is a mechanism that could explain progressive growth of relatively symmetrical lesions in elderly patients, many of whom have compromised circulation secondary to diseases that cause narrowing of the blood vessels such as hypertension and diabetes.

Our data are only for 1 time period and cannot be used to determine causality. We cannot exclude the possibility in some patients that sensitized, fibrotic blood vessels form small thrombotic lesions, leading to the formation of a series of small silent strokes in multiple locations. In that situation, the neuroinflammatory response involving MMP production may be secondary to the multiple strokes. Longitudinal

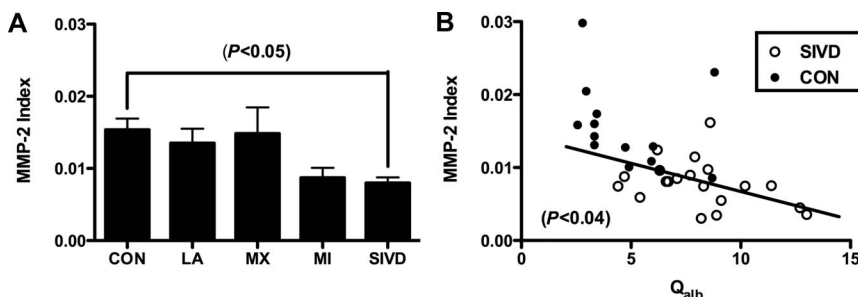


Figure 3. **A**, Matrix metalloproteinase-2 (MMP-2) index values plotted for each of the subgroups. Control values were mainly > 0.01 , whereas the SIVD group was significantly lower (Kruskal-Wallis with Dunn correction; $P < 0.05$). **B**, MMP-2 index showed a linear correlation with Q_{alb} for patients with SIVD ($P < 0.04$). SIVD indicates subcortical ischemic vascular disease; Q_{alb} , albumin index.

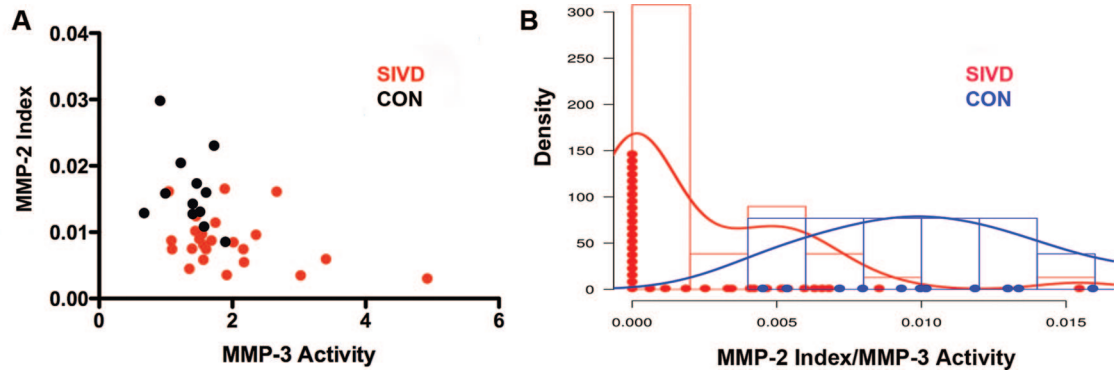


Figure 4. Relationship of matrix metalloproteinase-2 (MMP-2) index to MMP-3 activity for the SIVD and control groups. **A**, Scatterplot of MMP-2 index and MMP-3 activity shows that the control subjects clustered in the high MMP-2 index and low MMP-3 activity quadrant (black dots). The SIVD group clustered in the low MMP-2 index and high MMP-3 activity (red dots). **B**, A density plot of the ratio of MMP-2 index to MMP-3 activity shows that the 2 groups could be separated based on their means with a probability value of 0.0005. SIVD indicates subcortical ischemic vascular disease.

studies in larger populations will be necessary to resolve this important question.

Acknowledgments

Patient studies were done in the General Clinical Research Center at the University of New Mexico.

Sources of Funding

This work was supported by a grant from the National Institutes of Health to G.A.R. (R01 NS052305) and by a grant from Bayer Pharmaceutical Corporation.

Disclosures

E.C.-J. was a collaborator on grant R01 NS052305; S.T. was a collaborator on grant R01 NS052305; G.A.R. was Principal Investigator on grant R01 NS052305 as well as on a research grant from Bayer Pharmaceutical Corporation.

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