

**Correlation between MMP-9 and extracellular cytokine HMGB1 in prediction of
human ischemic stroke outcome**

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Abstract Ischemic stroke (IS) outcome predictors include clinical features, biochemical parameters and some risk factors. The relations between two main players in the ischemic brain, MMPs and HMGB1, were estimated in plasma of ischemic stroke patients stratified according to the Glasgow Outcome Scale and the Oxfordshire Community Stroke Project classification. IS patients exhibited higher plasma concentration of MMP-9 and the inflammatory cytokine HMGB1 compared with healthy controls. A full-blown correlation between MMP-9 activation and increased plasma MMP-9 concentration was observed in case of IS patients. A similar activity of MMP-2 and MMP-12 was characteristic of healthy volunteers and IS patients. In patients with ischemic stroke increased plasma levels of MMP-9 and HMGB1 are associated with a poor functional outcome and are significantly correlated with each other ($P=0.0054$). We suggest that diagnostic benefits will be obtained if plasma HMGB1 levels are measured for IS patients in addition to MMP-9.

Key words: human ischemic stroke, matrix metalloproteinases, HMGB1, gelatin zymography

1. Introduction

The pathogenesis of ischemic stroke is highly complex, involving inflammation, hemostasis, neuronal and glial injury. Matrix metalloproteinases (MMPs) are markers, amongst others, for the pathological IS processes, showing a significant association between their levels and the pathology. MMPs can broadly target almost all components of the mammalian central nervous system [1]. MMPs attack the extracellular matrix (ECM) around the blood vessels as well as the matrix around neurons, facilitating neural cell death. Several MMPs, especially MMP-9, have been implicated in the regulation of blood-brain barrier (BBB) permeability

and function during ischemic stroke [2]. In the early phase (hours to days) after cerebral ischemia, MMPs disrupt the blood-brain barrier, leading to BBB leakage, leukocyte infiltration, brain edema, and haemorrhage [3].

A number of reports have indicated that the High-mobility group box 1 protein (HMGB1) plays the role of a danger signal in the ischemic brain. It is involved in the pathogenesis of a variety of non-infectious inflammatory conditions including sterile injuries generated as a result of ischemia/reperfusion and stroke [4].

HMGB1 is a multipotent protein. It is generally accepted that it lives a double life: as an architectural transcription factor in the nucleus and as an extracellular signal. HMGB1 is passively released from all nucleated cells during necrosis and it signals to neighboring cells of ongoing damage [5]. Although apoptotic cells produce less HMGB1 than do necrotic cells, macrophage engulfment of apoptotic cells leads to greater HMGB1 release. HMGB1 can also be actively released from immune cells, including macrophages, monocytes, neutrophils, platelets and also from endothelial cells [6]. Macrophages release HMGB1 in response to various stimuli including oxidative stress. As an actively secreted protein from macrophages, HMGB1 can stimulate inactive immune cells to produce cascades of cytokines.

Ischemic injury causes tissue damage and around the ischemic core, inflammatory processes cause further damage in the penumbra. HMGB1 is rapidly released due to tissue damage and it is actively secreted around the ischemic core by activated immune cells. The HMGB1 content is high in neurons, Schwann cells, endothelial cells, monocytes and macrophages under these conditions [7]. All these cells can be source of extracellular HMGB1 after acute ischemic stroke and measurements of HMGB1 in blood at acute ischemic stroke can be considered as noninvasive tool for monitoring the condition.

Matrix metalloproteinases are secreted into the blood by a variety of connective tissue and proinflammatory cells, including fibroblasts, osteoblasts, endothelial cells, macrophages, neutrophils and lymphocytes [8].

The main objectives of this study are to test whether the profiles of matrix metalloproteinases MMP-2, MMP-9, MMP-12 and the extracellular cytokine HMGB1 change in the plasma of patients during the acute stage of the disease (the 24 hours following stroke onset), and whether they can provide prognostic information for stroke severity and pathophysiology. In addition we investigate the relationship between MMP-9 and HMGB1 as a functional outcome of IS. An evaluation of this relationship highlights HMGB1 and MMP-blocking agents as potential therapeutic tools for neuroprotection following IS.

2. Materials and Methods

2.1. Clinical study

Case subjects were selected from acute stroke patients admitted to the Sarajishvili Institute of Neurology and Neurosurgery (SINN). 42 eligible subjects (22 males and 20 females, average age of 69 ± 15 years) were selected from 70 patients with suspected acute stroke admitted to Clinical or Critical Care departments of the SINN. Reasons for exclusion included: final diagnoses other than stroke (7 cases), admission after 24 hours of stroke onset (4 cases), hemorrhagic stroke (10 patients) and patients' refusal to participate in the study (7 cases). All study subjects underwent the following investigations: detailed neurological examination (special stroke scales for evaluating the stroke severity and functional state were used according to the study protocol), CT, Extracranial Dopplerography, EKG and detailed

laboratory work-up including routine blood and urine analysis, coagulation tests, venous hematocrit and routine blood biochemistry tests (glucose and total cholesterol). Patients were clinically evaluated using the Glasgow Outcome Scale (GOS), the Glasgow Coma Scale (GCS), the Barthel-Rankin Scale and the Allen Scale. In addition, patients were stratified according to the Oxfordshire Community Stroke Project (OCSP) classification. The OCSP classification is widely used for stroke pathophysiology classification. This classification divides cerebral infarction into four categories: total anterior circulation infarction (TACI), partial anterior circulation infarction (PACI), lacunar infarction (LACI), and posterior circulation infarction (POCI). In our study, there were insufficient numbers of POCI cases for statistical analysis.

Healthy individuals were selected randomly from outpatients paying visits to the Polyclinic of the SINN and healthy donors were selected from the Blood Bank of Jo Ann's Medical Center (Tbilisi, Georgia). Controls were people without acute stroke/history of stroke and without current acute or chronic inflammatory illness. This study includes plasma samples from 32 healthy individuals (15 males and 17 females; average age of 63 ± 20 years). Blood samples were drawn in sterile tubes and then centrifuged for the further analyses of plasma. The study protocol was approved by the local ethics committee, and written informed consent was obtained from each participant or their relatives before inclusion in the study.

2.2. *Gelatin zymography*

Substrate zymography is the most sensitive assay for MMP detection. Plasma samples were analyzed on 12% (w/v) acrylamide gels containing 1.5 mg/ml gelatin in the presence of sodium dodecyl sulphate (SDS) under non-reducing conditions, in accordance with Gerlach

et al., [9] with some modifications. After electrophoresis the gels were washed in the Renaturing Buffer (50 mM Tris-HCl, pH 7.5; 2.5% w/v Triton X-100) to remove the SDS. Overnight incubation of the gel at 37°C in the Developing Buffer (50 mM Tris-HCl buffer, pH 7.5, 0.2 M NaCl, 5 mmol/L CaCl₂, and 0.2% Brij 35) allowed the reactivated enzyme to degrade the copolymerized substrate. Subsequently, the gels were stained with 0.05% Coomassie Brilliant Blue R-250 and the areas where the gelatin substrate has been degraded by gelatinases develop into white lines on a dark background. The loading corresponded to 1 µl of plasma. Gelatinase activity was confirmed as MMP (Zn²⁺- and Ca²⁺- dependent endopeptidases) since it was inhibited by 20 mM 1,10-phenanthroline, a zinc chelator, and by 5 mM EDTA, the broad-spectrum metal chelator (data not shown).

2.3. *Western blot method for the detection of total MMP-12 in plasma*

Western blotting with anti-MMP-12 antibodies (Cat.-No.: M-0807 Sigma, USA) was used to detect plasma MMP-12 in ischemic stroke patients and in normal plasma. Western blots were prepared from total plasma protein samples diluted x1:25 in Laemmli loading buffer and separated on 12% (w/v) SDS-polyacrylamide gels (normalized to 80 µg per lane) and blotted onto nitrocellulose Hybond ECL membrane (Amersham, USA). Membranes were blocked with 5% (w/v) Marvel in 1xPBS for 1 hr, then washed with 1xPBS and 0.1% Tween 20 and finally incubated with diluted antibodies (IgG) against human MMP-12. The primary antibodies (1:1000) were developed for 1 hr at RT, followed by secondary goat anti-rabbit IgG (1:1000) (Cat.-No.: A-9169 Sigma, USA) for 1 hr at RT. After further washing with 1xPBS and 0.1% Tween 20, chemiluminescent detection was performed using X-ray film (Kodak, USA). To be sure that the lines correspond to MMP-12, the human MMP-12 (Cat.-

No.: ab131994, Abcam, UK) and the ColorBurst electrophoresis marker (M.W. 8,000-220,000) (Cat.-No.: C1992 Sigma, USA) were used.

2.4. *ELISA method for the quantification of MMP-9 and HMGB1 in plasma*

MMP-9 and HMGB1 assay ELISA kits (Cat.-No.: BE59491 and ST51011 respectively, IBL International, Germany) based on monoclonal antibodies to human MMP-9 and polyclonal antibodies to HMGB1 were used to quantify MMP-9 and HMGB1 in plasma, following the manufacturer's instructions. The human MMP-9 levels ranged between 8.0 and 90.0 ng/ml for plasma samples from the apparently healthy donors. HMGB1 concentration in plasma of healthy donors is less than 1.4 ng/ml.

2.5. *Methods of analysis*

All values are expressed as means and medians by using Origin for Windows, version OriginPro8, and were analyzed using the Mann-Whitney *U* test (two-tailed). Correlations between variables were determined by Spearman rank test and Pearson rank test. Spearman rank correlation coefficient (r_s) provides a measure of how closely two sets of rankings agree with each other. Pearson correlation coefficient (r_p) is a measure of the strength of the association between the two variables. A P value <0.05 was taken to be of statistical significance; a P value <0.01 was taken to indicate a significant difference; a P value ≥ 0.05 was taken not to be of statistical significance.

3. Results

3.1. MMP-9 and MMP-2 in plasma following ischemic stroke

MMPs are synthesized and secreted as inactive proenzymes (zymogens) that subsequently become proteolytically cleaved and activated. The visualization of the pro-MMP-9 (MW=92 kDa) and active MMP-9 (MW=87 kDa) forms is presented in Fig. 1. Both forms of MMP-9 were present in the blood samples of control and IS patients. However, the MMP-9 zymograms show that there are significant differences in the ratio of pro-active and active MMP-9 forms in ischemic stroke patients compared to the control samples. In the case of IS patients the active MMP-9 form is increased significantly. As concerns the healthy volunteer controls, only in five studied subjects was an increase of the active MMP-9 form observed. As follows from Figure 1, MMP-2 is present in pro-active form (MW=72 kDa) in all cases. Activation of MMP-2 (to give an active form with MW=67 kDa) was not observed. The same profile of MMP-2 was observed in samples from healthy volunteers and from IS patients.

3.2. MMP-9 concentration in plasma following acute ischemic stroke. Relation to the clinical outcome and to the cerebral infarction category

MMP-9 plasma concentration, determined using monoclonal antibodies to human MMP-9, quantitatively estimates all forms of MMP-9 present in plasma: inactive, active and inhibited by forming a complex with the specific tissue inhibitor of metalloproteinases (TIMP).

Neurological impairment after IS refers to a broad group of disorders in which the central nervous system does not function properly and leads to some form of physical and mental problems. We classified patients according to GOS and compared the MMP-9 plasma

concentration in each category with the control. GOS evaluates clinical outcome on a scale from 1 to 5: scale point one constitutes the worst outcome (death), whereas point 5 represents the best score (recovery). The clinical outcome was evaluated at hospital discharge and one month after ischemic stroke. The data presented in the Fig. 2A shows that the negative outcome is significantly correlated with the increased MMP-9 concentration in plasma, estimated 24 hr after IS onset.

The MMP-9 plasma concentration in each category of the Oxfordshire Community Stroke project classification was also compared with the controls (Fig. 2B). The total anterior circulation infarction (TACI) category is likewise significantly correlated with the increased MMP-9 concentration in plasma estimated 24 hr after IS onset.

3.3. MMP-9 concentration and activation at ischemic stroke

A correlation between MMP-9 activation and increased plasma MMP-9 concentration was observed for the IS patients in our study. The increased MMP-9 concentration observed for some control volunteers also corresponds with MMP-9 activation. The data are presented in Table 1.

3.4. Plasma extracellular cytokine (HMGB1) level in ischemic stroke patients. Relationship to clinical outcome and to cerebral infarction category

It was reported that serum HMGB1 levels, analyzed by Western blotting, were significantly elevated in both cerebral ischemia subjects and in myocardial ischemia subjects [10]. Our

results are based on the estimation of plasma HMGB1 concentration by ELISA in ischemic stroke subjects and in control subjects.

We have considered the plasma HMGB1 concentration in relation to clinical outcome, and a correlation between increased HMGB1 concentration and poor outcome has been observed. The data are presented in the Fig. 2C. The HMGB1 plasma concentration in each category of the Oxfordshire Community Stroke Project classification was compared with the controls and this is presented in the Fig. 2D. As follows from Fig. 2D, total anterior circulation infarction (TACI) is significantly correlated with increased HMGB1 concentration in the plasma of IS patients.

3.5. The correlation between MMP-9 activation and levels of the extracellular cytokine HMGB1 at acute ischemic stroke

Our data confirm that activated MMP-9 can be considered a good marker for a poor outcome (GOS) at IS within the early phase of ischemic stroke development. As follows from the above results, increased HMGB1 can be also considered as a marker for poor outcome (GOS) at IS within the first 24 hr of ischemic stroke development. This is why we have examined the links between MMP-9 and HMGB1 levels for the different functional outcomes accord to GOS classification. In patients with ischemic stroke the increased plasma MMP-9 and HMGB1 levels are associated with a poor functional outcome and significantly correlated with each other ($P < 0.05$). The results are presented in the Fig. 3A. In case of a fairly good functional outcome (Fig. 3B), no correlation has been observed between MMP-9 and HMGB1 levels in plasma ($P > 0.05$).

3.6. MMP-12 in plasma at ischemic stroke

MMP-12 is expressed in alveolar macrophages. The mean MMP-12 concentration in the plasma of healthy subjects is 1.2 pg/ml. A commercially available multiplex beads immunoassay has been used to estimate plasma MMP-12 concentrations in moyamoya disease patients and in controls [11]. It was showed that even using this immunoassay, MMP-12 content in controls is out of range of detection but is detectable in moyamoya disease patients at ~9 pg/ml. We used Western blotting with anti-MMP-12 antibodies for the examination of plasma MMP-12 in ischemic stroke patients. MMP-12 is characterized by a MW 60 kDa (zymogenic form) and a set of active forms have MWs ranging from 50/45 to 25 kDa can be observed as the result of auto-proteolytic activity (Fig. 4). The main upper band corresponds to molecular weight (MW) 45 kDa, and there are several lines under it with the minimum at MW 25 kDa. The MMP-12 zymogenic form is not detectable in the plasma samples, consistent with the high auto-proteolytic properties of MMP-12.

Another important feature of MMP-12 is its ability to activate other MMPs *in vivo* [12]. To find out if the MMP-9 in IS samples is activated by MMP-12, the IS samples characterized by the prominent MMP-9 active form (IS17, IS7, IS11) and the control samples, characterized by low levels of MMP-9 active form (C6, C7, C16), have been analysed for their MMP-12 quantity (Fig.4). The intensity of the main band (45 kDa) for the studied samples was very similar, which points to an equal quantity of MMP-12 in plasma for both types of samples. Therefore, we can conclude that MMP-9 activation at acute ischemic stroke is not the result of MMP-12 action.

4. Discussion

MMPs can degrade many matrix components, however under normal physiological conditions their proteolytic enzyme activities are strictly controlled via gene expression, proenzyme activation and inhibition by tissue inhibitors of metalloproteinases and enzyme degradation [13]. In pathological conditions this equilibrium can be shifted towards increased MMP activity leading to tissue and extracellular matrix degradation [14]. Among early serological markers of substantial brain insult and poor outcome, MMPs deserve much attention since a growing body of evidence points to their key role in the ischemic cascade. The activation of MMPs has been implicated as a negative prognostic factor in human stroke because MMPs are able to cleave type IV collagen, laminin and fibronectin, the major components of the basal lamina around cerebral blood vessels. Plasma MMP-9 concentration was identified as a predictor of cardiovascular mortality in patients with coronary artery disease [15]. A strong correlation was also found between MMP-9 and MMP-13 with diffusion-weighted image (DWI) lesion expansion in cases of acute ischemic stroke [16]. Other clinical studies have shown that MMP-9 expression in acute cardioembolic stroke is related to hemorrhagic transformation, whereas its levels are associated with neurological deficit, middle cerebral artery occlusion and infarct volume [17,18]. The elevated level of MMP-9 mRNA is a predictor of poor outcome and mortality in stroke [19].

In the present study, the increased level of MMP-9 after acute IS is observed, and the strong correlation between increased plasma MMP-9 concentration and MMP-9 activation was noted for IS patients. The activated MMP-9 can be considered as an effective marker for negative outcome (GOS) at IS within the early phase of ischemic stroke development. Additionally, IS pathophysiology classification of the patients in accord with OCSF revealed

that MMP-9 concentration increased only in the plasma of patients with total anterior circulation infarction.

Plasma HMGB1 level is considered as an independent predictor of long-term clinical outcomes of IS [20]. An increase in plasma HMGB1 concentration, persisting for 30 days, is associated with stroke [21]. In the present study, the increased level of plasma HMGB1 after acute IS is observed within 24 hr of stroke onset, and the correlation between increased HMGB1 concentrations and IS outcome has been detected.

It is well known that the use of a combination of several different markers may help to minimize the variability of any individual marker. For this reason we made the first study of the relation between MMP-9 and the proinflammatory cytokine HMGB1 following acute IS. A strong correlation was detected between increased MMP-9 and HMGB1 levels and with a poor outcome (death or functional decline). A possible cause of the synchronized increase of plasma MMP-9 and HMGB1 levels could be secretion from neutrophils, as MMP-9 is a neutrophil-derived blood MMP and a high quantity of HMGB1 characterizes these blood cells. HMGB1 can be actively released from neutrophils in response to development of prooxidant and proinflammatory conditions that develop at IS [22]. The inflammatory activity of HMGB1 is dependent upon the oxidation status of cysteine 106 residing within the B box, a region that is critical for stimulating cytokine release and inflammation [23]. Mechanisms upregulating MMP expression remain largely unknown but numerous studies now suggest that reactive oxygen species also regulate MMP activity [24,25].

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Table 1. Characteristics of MMP-9 activation in plasma of selective Controls (C) and Ischemic Stroke patients (St) within the first 24 hr of IS onset

Controls (Healthy Volunteers)	MMP-9 ng/ml (9-80 ng/ml norm)	Active MMP-9	Ischemic Stroke (Patients)	MMP-9 ng/ml (9-80 ng/ml norm)	Active MMP-9
C2	32.9	-	St2	241	+
C6	35.9	-	St3	101	+
C7	44	-	St4	551	+
C9	151	+	St7	172	+
C10	134	+	St8	71.3	-
C12	108	-	St9	25.7	-
C13	131	-	St11	428	+
C14	161	+	St13	269	+
C15	89.3	-	St14	51	-
C16	66.5	-	St15	49	-
C17	143	+	St17	535	+
C18	125	+	St18	235	+
C19	49.7	-	St21	105	+
C20	58	-	St22	66	-
C21	9	-	St27	585	+

Figure captions

Figure 1.

Representative zymogram of plasma sample corresponding to healthy volunteers (C - controls), ischemic stroke patients (IS), and marker (M). The positions of pro-MMP-9, activated MMP-9, and pro-MMP-2 as the achromatic zones are indicated by arrows. A mixture of MMP-9 and MMP-2 containing gelatinase was used as a marker. The loading corresponds to 1 μ l of plasma, normalized to the concentration of total plasma proteins.

Figure 2.

Plasma levels of MMP-9 and HMGB1 in controls and ischemic stroke patients stratified according to GOS and the OCSF classification. Glasgow Outcome Scale (GOS): IS (1 to 3) – Poor outcome (death and functional decline); IS (4) – Fairly good outcome (moderate disability); IS (5) – Good outcome (recovery). The OCSF classification includes: total anterior circulation infarction (TACI), partial anterior circulation infarction (PACI), lacunar infarction (LACI). Values are analyzed using the Mann-Whitney *U* test. A *P* value <0.05 is taken to be of statistical significance; a *P* value <0.01 is taken to indicate a significant difference; a *P* value \geq 0.05 is taken not to be of statistical significance.

Figure 3.

Correlations between levels of plasma HMGB1 and MMP-9 in IS patients with different functional outcomes. Correlations were determined by combining data from 42 patients using Spearman and Pearson rank correlation analysis. (A) IS (1 to 3) – Poor outcome (Spearman $r=0.71$, $P=0.046$; Pearson $r=0.86$, $P=0.0054$). (B) IS (4 and 5)- Favorable outcome (Spearman $r=0.037$, $P=0.85$; Pearson $r=-0.13$, $P=0.52$)

Figure 4.

Western blot, stained with anti-MMP-12, demonstrating the same quantity of MMP-12 in plasma of IS patients and in healthy volunteers and the autoproteolytic properties of MMP-12 in plasma samples. MMP-12 pro-enzyme was used as a standard. The ColorBurst electrophoresis marker was used as a MW marker. C – controls; IS – ischemic stroke patients; M – MW marker, St- MMP-12 pro-enzyme.

Figure 1.

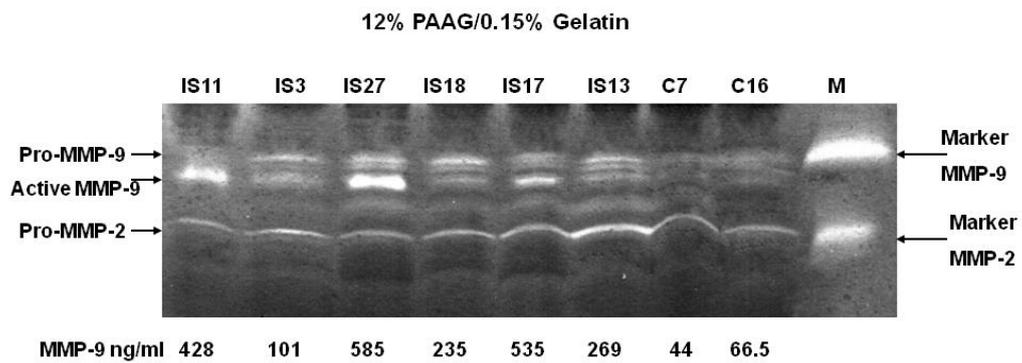


Figure 2.

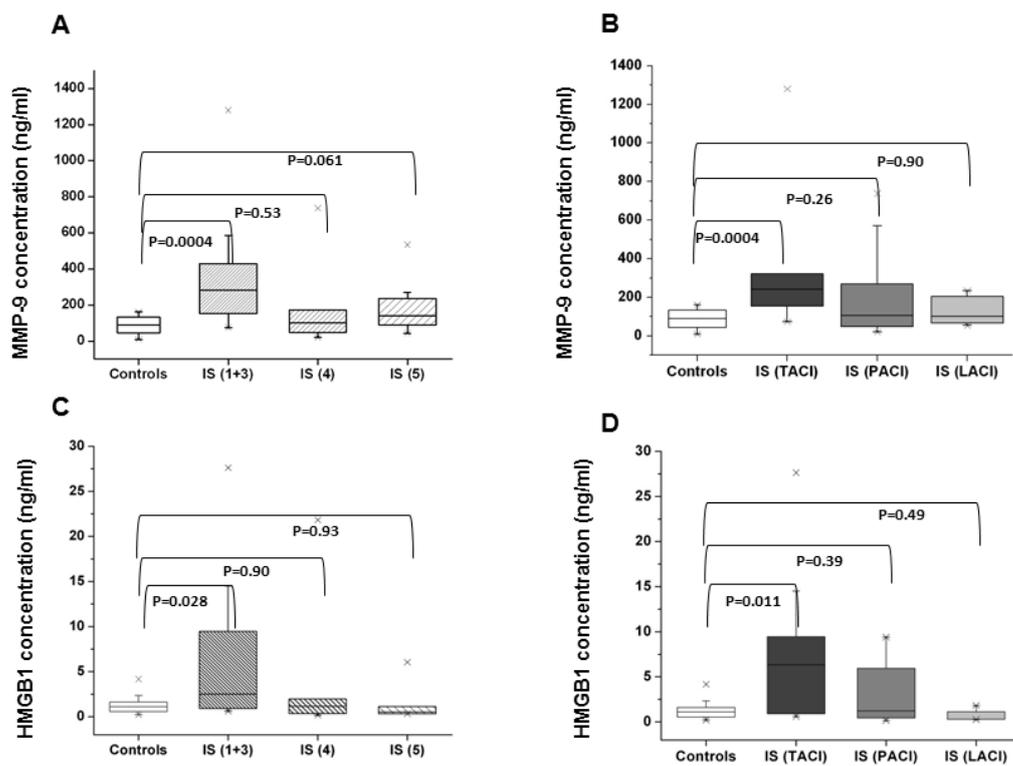


Figure 3.

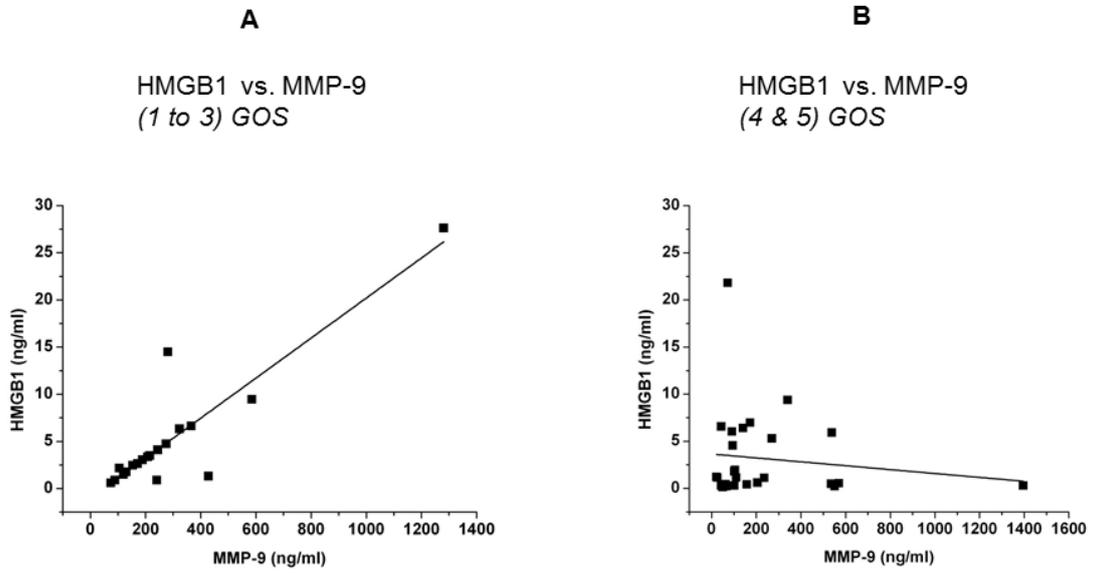


Figure 4.

