

# Associations between serum L-arginine and ficolins in the early phase of acute ischemic stroke – A pilot study

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**Introduction:** Activation of both the L-arginine and the lectin pathway contributes to the pathophysiology and the outcome of acute ischemic stroke (AIS). However, the interplay between the two systems has not yet been examined. **Methods:** A total of 44 patients with AIS were recruited into this study. Serial measurement of serum L-arginine, asymmetric and symmetric dimethylarginine (ADMA, SDMA), and hsCRP, ficolin-2, ficolin-3, MAP-1, MASP-3 and mannose-binding lectin (MBL) were analyzed within 6 h after onset of stroke and 72 h later. Outcomes were assessed as National Institutes of Health Stroke Scale (NIHSS) worsening by 24 h, poststroke infection, and death by 1 month. **Results:** In the hyperacute stage of AIS, ficolin-3, MAP-1 and MBL were positively correlated with L-arginine within 6 h after onset of symptoms ( $p < 0.05$  respectively). Significantly lower ficolin-3 and MASP-3 levels were found at 72 h in patients, who developed post-stroke infection after day 4, when compared to patients without post-stroke infections ( $p = 0.03$  and  $p = 0.009$ ). At 72 hours, ficolin-3 levels negatively correlated with S100B ( $p = 0.01$ ). Ficolin-3 at 72 post-stroke hours remained an independent predictor of post-stroke infection, while only hsCRP was an independent predictor of 30-day mortality. **Conclusion:** Early consumption of ficolin-3 is associated with complications such as post-stroke infections. In the hyperacute phase of AIS, the positive correlation between ficolins and the NO donor L-arginine may reflect the protective role of L-arginine presumably by improving the cerebral microcirculation in a prothrombotic environment induced by complement activation.

**Key Words:** Ischemic stroke—L-arginine—Ficolin—Mannose-binding lectin—Infection—Outcome

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## Introduction

Post-stroke inflammation and immunodepression are mediated by systemic immune cells, endothelial cells, microglia, and neurons.<sup>1–3</sup> Both infection and inflammation can influence the outcome of ischemic stroke.<sup>4–6</sup> Markers for immunodepression, inflammation and infection are being intensively investigated.<sup>7–9</sup> Activation of the complement system is a key element in the ischemic cascade resulting in an unfavorable outcome due to secondary brain injury.<sup>10</sup> Several studies have explored the temporal profile of the lectin pathway molecules in the sera of patients with acute ischemic stroke (AIS) showing: (i) a decrease in concentration of ficolins in the very early phase of stroke; (ii) unchanged concentration during the subacute phase; (iii) an inverse correlation between ficolin-3 and astrocyte-derived S100B in the follow-up samples suggesting that greater size of the infarct results in higher consumption of ficolin-3.<sup>11,12</sup> Besides, the low

levels of ficolins were found to be associated with an unfavorable prognosis in AIS.<sup>11,13</sup>

The mannose-binding lectin (MBL) pathway also contributes to the pathological processes. A deficiency of MBL is associated with smaller infarction size and a more favorable outcome.<sup>14</sup> In focal cerebral ischemia model, MBL was a key player in the pathogenesis of ischemic injury suggesting that MBL inhibition may be a relevant therapeutic target in humans with a wide therapeutic window of application.<sup>15,16</sup>

Besides complement activation, other inflammatory processes are also known to contribute to the pathogenesis and outcome of the ischemic stroke.<sup>1</sup> Elevated serum levels of L-arginine pathway molecules were observed in the very acute phase of ischemic stroke indicating a more pronounced endothelial dysfunction compared with asymptomatic significant carotid stenosis.<sup>17</sup> Moreover, an increased level of the NO donor L-arginine was found in patients with AIS on admission.<sup>17</sup> A transient elevation of the L-arginine/asymmetric dimethylarginine (ADMA) ratio was observed at the critical 24 post-stroke hours suggesting the protective role of L-arginine, and changes in the L-arginine pathway were predictive of post-stroke infections.<sup>17</sup> In addition, the concentration of L-arginine and ADMA/ symmetric dimethylarginine (SDMA) differentially correlated with thrombo-inflammation in the hyperacute phase of ischemic stroke and such correlations were independently associated with post-stroke infection.<sup>18</sup>

Besides, CRP is an independent predictor of cerebrovascular events and prognosis after stroke. CRP is an important molecule in the progression of cerebral tissue injury.<sup>19</sup> Complement activation and elevated CRP levels were independently associated with the clinical severity and different outcome measures of ischemic stroke, indicating their additive effect.<sup>6</sup> An early elevation of CRP

inversely correlated with the concentration of L-arginine suggesting that L-arginine may play a protective role and the low L-arginine levels are associated with a higher concentration of CRP, a risk factor for ischemic stroke.<sup>17</sup> In contrast, further elevation of CRP by 72 hours was associated with high concentration of ADMA,<sup>17</sup> contributing to brain injury by reducing cerebral blood flow and facilitating excitotoxic neuronal death.<sup>20</sup> Early elevation of CRP was a strong predictor of mortality, while late elevation predicted post-stroke infection as an early subclinical sign of infection.<sup>21</sup>

Here, we aimed to explore the interplay between the lectin and L-arginine pathway and their impact on post-stroke infection and outcome.

## Methods

This research has been complied with all the relevant national regulations, institutional policies, and in accordance the tenets of the Helsinki Declaration. The study was approved by the local ethics committees. Written informed consent was obtained from all individuals included in this study.

### Subjects

Patients with AIS were prospectively examined (Table 1). Part of this cohort overlapped with a previously published cohort.<sup>11</sup> Patients were enrolled upon the first occurrence of acute ischemic stroke only; all patients had neuroimaging (most of them brain MRI, but at least cranial CT). All patients with definite acute clinical symptoms were enrolled regardless of etiology i.e. lacunar or territorial infarct caused by thrombosis or emboli. Exclusion criteria were infectious diseases, fever < 4 weeks before stroke, an elevated white blood cell

**Table 1.** Demography and clinical factors.

	all patients n=44	with infection n=7	without infection n=37	P
Age (year)	67±10	66±6	72±9	0.692
Male	21	3	18	0.778
BMI	27.5±5.8	31.1±8.5	26.8±5.3	0.498
Smoking, %	7 (16)	1 (14)	6 (16)	0.755
Hypertension, %	36 (81)	5 (71)	31 (84)	0.589
Diabetes mellitus, %	8 (18)	1 (14)	7 (19)	0.196
Dyslipidemia, %	18 (41)	3 (43)	15 (41)	0.847
NIHSS on admission	10.4±5.3	14.4±6.9	9.5±4.6	0.072
NIHSS day 2	10.4±6.0	13.0±6.2	9.9±6.1	0.401
NIHSS on discharge	10.5±8.4	19.7±5.0	6.5±6.0	0.001
Death, %	5 (11)	5 (71)	0	<0.001
Antiplatelet therapy, %	25 (57)	5 (71)	20 (54)	0.460
Statin therapy, %	18 (41)	3 (43)	15 (41)	0.847

BMI=body mass index; NIHSS=National Institute of Health Stroke Scale. Data are shown as mean±SD or absolute number (%). Chi-square test and Mann-Whitney test.

(WBC), erythrocyte sedimentation rate (ESR), high-sensitivity CRP (hsCRP, cut-off value < 5 mg/L), procalcitonin on admission (cut-off value < 0.05 ng/mL), positive chest X-ray, hemorrhagic stroke defined by an acute cranial CT scan, and those who declined to participate in the study.<sup>11,21</sup> An evidence-based guideline<sup>22</sup> was followed to detect post-stroke infectious complications (in short, physical and laboratory measures including WBC, ESR, hsCRP, PCT, fever, abnormal urine, chest X-ray or positive cultures). Such complications, predominantly pneumonia and urinary tract infection occurred on the 5th day as an average. The severity of stroke was assessed each day by National Institutes of Health Stroke Scale (NIHSS).<sup>23</sup> Outcome measures were the worsening of NIHSS by 24 h, post-stroke infections, and 30-day mortality.

### Biomarkers

Serial measurement of serum L-arginine, ADMA and SDMA, and serum hsCRP, ficolin-2, ficolin-3, MAP-1, MASP-3 and MBL were analyzed within 6 h after onset of stroke and 72 hours later. Ficolin-2 and ficolin-3, mannose-binding lectin (MBL), MBL/Ficolin associated Protein-1 (MAP-1), and MBL associated serine protease-3 (MASP-3) were determined by ELISA-based methods at the Laboratory of Molecular Medicine, Department of Clinical Immunology, Rigshospitalet, Copenhagen, Denmark as described earlier.<sup>24</sup> Serum S100B concentrations were measured by an ELISA method (BioVendor, Modrice, Czech Republic). Serum concentrations of hsCRP were measured by particle-enhanced immunoturbidimetric assay, using an automated laboratory analyzer (Roche Cobas Integra 400, Basel, Switzerland). The serum concentration of L-arginine, ADMA and SDMA were determined by High Pressure Liquid Chromatography (HPLC), at the Department of Applied

Chemistry, University of Debrecen, Hungary. The determination of L-arginine and its derivatives was described earlier.<sup>17,18</sup>

### Statistical analysis

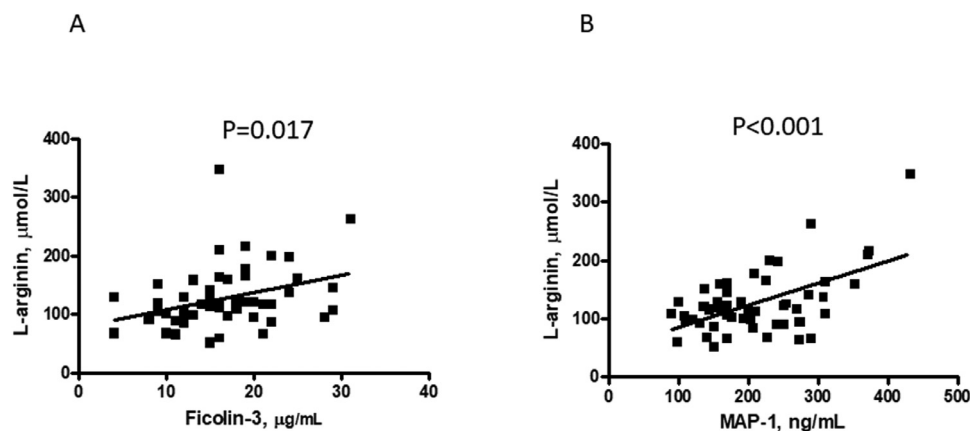
Statistical analyses were performed using SPSS 20.0 software (IBM Corp., Armonk, NY). Comparison of categorical variables between the groups was performed by chi-square test. Normal distribution of continuous variables was checked by Kolmogorov-Smirnov test. Mann-Whitney *U* test was used to compare not normally distributed parameters. Correlation analysis was performed calculating Spearman's correlation coefficient (*r*). Binary logistic regression including baseline variables (age, gender, BMI, hsCRP, S100B, L-arginine and lectin pathway molecules) was used to explore independent predictors and ROC analysis was performed to calculate the cut-off value. Results were considered significant, if *p* < 0.05.

## Results

A total of 44 patients (mean age: 67, SD: 10 years, male: 21) (AIS) were recruited to this study. The demography and clinical parameters are shown in [Table 1](#).

### L-arginine and lectin pathway

In the hyperacute stage of AIS, ficolin-3, MAP-1 ([Fig. 1 \(A\) and \(B\)](#)) and MBL were positively correlated with L-arginine ( $r=0.331$ ,  $p=0.017$ ,  $r=0.557$ ,  $p<0.001$  and  $r=0.419$ ,  $p=0.02$  respectively), nevertheless neither ADMA nor SDMA correlated with any lectin pathway molecules within 6 h after onset of symptoms ([Table 2](#)). These correlations between ficolin-3, MAP-1, MBL and L-arginine disappeared by 72 post-stroke hours, but MBL showed an inverse correlation with ADMA ( $r= -0.397$ ,  $p=0.04$ ) and



**Fig. 1.** Correlation between L-arginine and ficolin-3 (A), and L-arginine and MAP-1 (B) in patients with acute ischemic stroke within 6 h after stroke onset. (A) Positive correlation between serum L-arginine ( $\mu\text{mol/L}$ ) and ficolin-3 ( $\mu\text{g/mL}$ ) concentrations on admission (scatter plot diagram, Spearman correlation,  $r=0.331$ ,  $p=0.017$ ). (B) Positive correlation between serum L-arginine ( $\mu\text{mol/L}$ ) and MAP-1 (ng/mL) concentrations on admission (scatter plot diagram, Spearman correlation,  $r=0.557$ ,  $p<0.001$ )

**Table 2.** Correlation among L-arginine, S100B and molecules of the lectin pathway on admission

	L-arginine	<i>p</i>	S100B	<i>p</i>
Ficolin-2	0.276	0.219	-0.216	0.182
Ficolin-3	0.398	0.03	-0.307	0.05
MAP-1	0.519	0.004	0.160	0.324
MASP-3	0.262	0.170	0.094	0.563
MBL	0.419	0.02	-0.082	0.613
hsCRP	-0.190	0.364	0.194	0.265

MBL=mannose-binding lectin; MAP-1= MBL/Ficolin associated Protein-1; MASP-3=MBL associated serine protease-3; hsCRP= high-sensitivity C reactive protein. Spearman correlation.

S100B ( $r=-0.434$ ,  $p=0.006$ ), while ficolin-3 negatively correlated only with S100B ( $r=-0.397$ ,  $p=0.01$ ) (Table 3).

#### C-reactive protein and L-arginine and lectin pathway

The serum concentration of hsCRP showed a positive correlation with L-arginine ( $r=0.485$ ,  $p=0.01$ ) and S100B ( $r=0.428$ ,  $p=0.02$ ) at 72 post-stroke hours in the total population (Table 2). In addition, hsCRP showed a negative correlation ( $r=-0.435$ ,  $p=0.01$ ) with ficolin-3 levels at 72 h.

#### Post-stroke infection

Patients were dichotomized into two subgroups: with ( $n=7$ ) and without post-stroke infection ( $n=37$ ). The stroke severity based on NIHSS at 24 h was significantly higher in patients with post-stroke infection compared to those without such complication ( $19.7\pm 5.0$  vs.  $6.5\pm 6.0$ ,  $p=0.001$ ). Post-stroke infection was developed in 7 patients on the average 5th post-admission day. Five of them died due to severe infection (predominantly pulmonary complication).

Significantly lower ficolin-3 and MASP-3 levels were found at 72 h in patients, who developed post-stroke infection later, when compared to patients without post-stroke infections ( $p=0.03$  and  $p=0.009$ ) (Figure 2(A) and (B)). In addition, MASP-3 inversely correlated with NIHSS assessed at hospital discharge ( $r=-0.482$ ,  $p=0.002$ ). A reduced serum concentration of ficolin-3 with a cut-off value of  $<13.3$   $\mu\text{g}/\text{mL}$  measured at 72

post-stroke hours was found a predictor of infection, but not of death (sensitivity: 76%; specificity: 78%, AUC: 0.753;  $p=0.03$ ).

#### Independent predictors of post-stroke infection

Based on multiple regression analysis including baseline variables, only ficolin-3 with a cut-off value of  $<13.3$   $\mu\text{g}/\text{mL}$  (sensitivity: 76%; specificity: 72%, AUC: 0.753;  $p=0.03$ ) measured at 72 post-stroke hours was a weak independent predictor of post-stroke infection (OR: 0.73, 95% Confidence Interval: 0.53–1.00,  $p=0.05$ ), while the independent predictor of 30-day mortality (OR: 1.05, 95% Confidence Interval: 1.002–1.100,  $p<0.05$ ) was only hsCRP at 72 with a cut-off value of  $>39.6$   $\text{mg}/\text{L}$  (sensitivity: 80%; specificity: 82%, AUC: 0.830;  $p=0.02$ ).

## Discussion

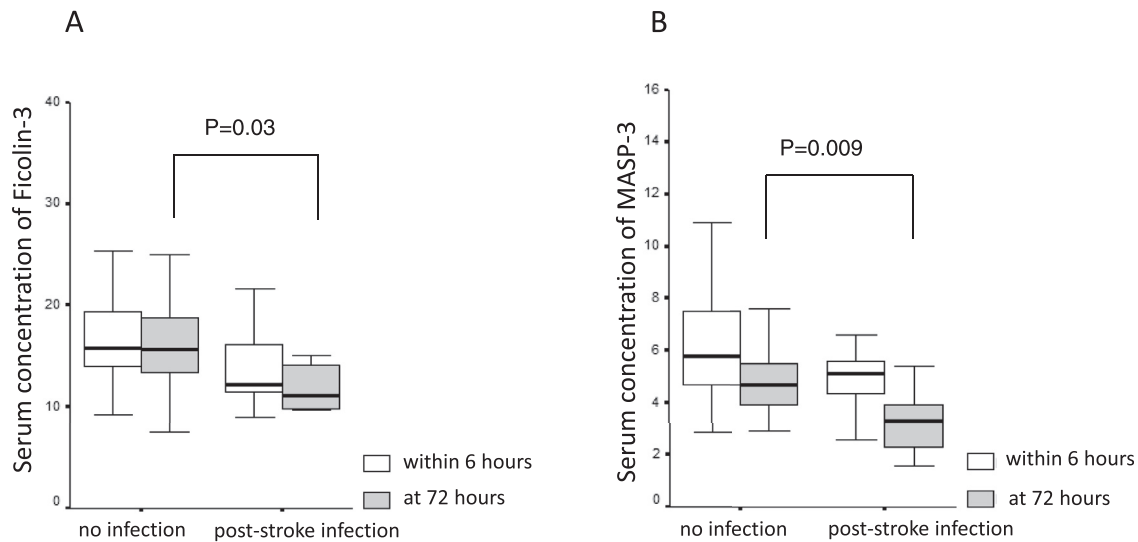
Here, we examined association between the L-arginine and lectin pathway molecules in the sera of patients with AIS within the first 72 post-stroke hours.

In the hyperacute phase of AIS, we observed a positive correlation between ficolin-3, MAP-1, MBL and the NO donor L-arginine reflecting the protective role of L-arginine presumably by improving the cerebral microcirculation in a prothrombotic environment induced by complement activation.<sup>25</sup> Beside initiating activation of complement via the lectin pathway, ficolins may trigger activation of the immune system by production of nitric oxide (NO) by macrophages, thus limiting the infection and concurrently orchestrating the subsequent adaptive immune response.<sup>26</sup> In acute coronary syndrome, the kinetics of oxidized low-density lipoprotein receptor-1 (LOX-1), a lectin like molecule, was described with an acute increase up to 12 post-event hour and a subsequent decrease, while NOx derived from the L-arginine-nitric oxide pathway, decreased promptly suggesting an impaired NO metabolism during acute ischemic coronary event.<sup>27</sup> Based on these observations, we assume that an increased availability of the L-arginine might be a counteracting response in the very early phase of ischemic stroke providing the following beneficial effects via restoration of NO production; (i) regulation of the vascular tone; (ii) playing a role as a cytotoxic

**Table 3.** Correlation between L-arginine, ADMA, S100B and lectin pathway molecules at 72 h.

	L-arginine	<i>p</i>	ADMA	<i>p</i>	S100B	<i>p</i>
Ficolin-2	0.058	0.774	0.155	0.439	-0.301	0.063
Ficolin-3	-0.104	0.605	-0.192	0.337	-0.397	0.012
MAP-1	0.167	0.404	0.211	0.290	-0.093	0.575
MASP-3	-0.173	0.389	-0.081	0.689	-0.294	0.069
MBL	0.059	0.771	-0.397	0.040	-0.434	0.006
hsCRP	0.485	0.016	0.089	0.994	0.428	0.018

ADMA=asymmetric dimethylarginine; MBL=mannose-binding lectin; MAP-1= MBL/Ficolin associated Protein-1; MASP-3= MBL associated serine protease-3; hsCRP= high-sensitivity C reactive protein. Spearman correlation.



**Fig. 2.** Ficolin pathway molecules in patients with or without post-stroke infections. (A) Serum ficolin-3 concentration on admission (white boxes) and 72 post-stroke hours (grey boxes) are compared in patients without and with post-stroke infection. (box-plot diagram, Mann–Whitney U test). (B) Serum MASP-3 concentration on admission (white boxes) and 72 post-stroke hours (grey boxes) are compared in patients without and with post-stroke infection. (box-plot diagram, Mann–Whitney U test)

effector in the immune system; (iii) acting as an intercellular neurotransmitter in the nervous system.

In the subacute phase of ischemic stroke, significant differences were found in the serum concentration of both, ficolin-3 and L-arginine by 72 h showing that clinically more severe AIS (NIHSS $\geq$ 16) was associated with lower ficolin-3<sup>11,17</sup> and higher L-arginine serum levels. It seems reasonable, that the ficolin-3 induced NO generation is exhausted in patients with worsening stroke despite a higher L-arginine supply in the subacute phase. A higher NIHSS score at 24 h reflects an early progression of AIS, thus the opposite change in serum ficolin-3 (decrease between 6 and 72 h) and L-arginine levels (increase between 6 and 72 h) may also suggest that higher infarct size is accompanied by further consumption of ficolin-3, which is presumably antagonized by a further L-arginine availability. Similarly, MBL, another component of the lectin pathway, showed negative correlation with ADMA an indicator of endothelium dysfunction, and also S100B, an indicator of infarct size suggesting an association between extended brain tissue injury and increased consumption of MBL by post-stroke 72 h. In accordance with our previous data, the S100B protein reaches its peak serum concentration at 72 h after the onset of stroke.<sup>21</sup> In addition, high plasma concentration of ADMA, an endogenous inhibitor of NO synthase, was observed in patients with silent brain infarcts on MRI.<sup>28</sup>

Consumption of ficolin-3 and MASP-3 by 72 h was also associated with complications such as post-stroke infections. Recently MASP-2 was reported playing a pivotal role in driving tissue injury and unfavorable outcomes in mouse models of ischemic brain injury.<sup>29</sup> In our earlier studies, low ficolin-3 and elevated hsCRP concentrations

were independently predictive for worse outcome.<sup>10,11</sup> Our finding, that ficolin-3 at 72 post-stroke hours was found to be an independent predictor of post-stroke infection, is in accordance with those observations that ficolin-3 has the highest post-stroke concentration and the greatest complement-activating capacity among the lectin pathway initiators.<sup>30</sup> The fact, that the independent predictor of 30-day mortality was only CRP, but not ficolin-3, suggests that they reflect two different pathways of inflammation contributing to the outcome of stroke.<sup>11,18,31</sup>

Although the sample size is small, our findings may suggest that both the lectin and L-arginine pathway plays a crucial role in post-ischemic stroke pathology including immunodepression leading to a poor outcome. Limitations of this study should be also mentioned: (i) the size and localization of the infarct could have been more accurately determined by follow-up MR imaging instead of S100B; (ii) the functional outcome based on the modified Rankin Scale was not assessed here; and (iii) it would have been interesting to clarify the recovery time of the lectin pathway by a follow-up study. However, the detailed mechanisms remain largely unknown, therefore larger studies will be required to establish the clinical implications of our finding.

### Declaration of Competing Interest

None declared.

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### Authors contributions

All the authors have accepted responsibility for the entire content of this submitted manuscript and approved submission.

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