Procarboxypeptidase U (proCPU, TAFI, proCPB2) in cerebrospinal fluid during ischemic stroke is associated with stroke progression, outcome and blood-brain barrier dysfunction

Procarboxypeptidase U (proCPU, TAFI, proCPB2) in cerebrospinal fluid during ischemic stroke is associated with to stroke progression, outcome and blood-brain barrier dysfunction

Mertens Joachim C[*], Leenaerts Dorien[*], Brouns Raf[†], Engelborghs Sebastiaan[‡,§], Ieven Margareta[¶], De Deyn Peter P[‡,§], Lambeir Anne-Marie[*], Hendriks Dirk[*].

[*]Laboratory of Medical Biochemistry, Department of Pharmaceutical Sciences, University of Antwerp, Antwerp, Belgium.

[†] Department of Neurology, Universitair Ziekenhuis Brussel, Brussels, Belgium.

[‡] Laboratory of Neurochemistry and Behavior, Institute Born-Bunge, University of Antwerp, Antwerp, Belgium.

[§] Department of Neurology and Memory Clinic, Hospital Network Antwerp (ZNA) Middelheim and Hoge Beuken, Antwerp, Belgium.

[¶] Department of Microbiology, University Hospital Antwerp, Edegem, Belgium.

Running head: ProCPU in CSF of acute ischemic stroke patients.

Total word count of the abstract: 246

Total word count of the body text: 2031

Correspondence: Dirk Hendriks, Laboratory of Medical Biochemistry, University of Antwerp, Universiteitsplein 1, 2610 Wilrijk, Belgium.

Tel.: +32 3 265 27 27; E-mail: dirk.hendriks@uantwerpen.be

Essentials

- Little is known of procarboxypeptidase U (proCPU) levels in CSF of stroke patients.
- ProCPU levels were investigated in CSF of controls and non-thrombolyzed acute ischemic stroke patients.
- ProCPU is elevated in CSF of stroke patients compared to controls.
- ProCPU in CSF correlates with stroke progression, outcome, and blood-brain barrier dysfunction.

Keywords

Cerebral Infarction

Procarboxypeptidase U

Carboxypeptidase B2

Cerebrospinal Fluid

Blood-Brain Barrier

Abstract

Background: Procarboxypeptidase U (proCPU, TAFI, proCPB2), the zymogen of CPU which is a potent antifibrinolytic enzyme and a modulator of inflammation has previously been investigated in plasma of stroke patients, but so far, no information on the proCPU levels in cerebrospinal fluid (CSF) during acute ischemic stroke (AIS) is available.

Objectives: This case-control observational study investigates proCPU in CSF of AIS patients compared to controls with an intact blood-brain barrier (BBB) and evaluates the relationship of CSF/plasma proCPU ratios with stroke parameters.

Methods: A sensitive HPLC-based enzymatic assay was used to determine proCPU levels in CSF of non-thrombolyzed patients in the hyperacute phase (<24h after onset) of AIS (n=72). Individuals (n=32) without stroke, an intact BBB and no apparent abnormalities in biochemical and microbiological tests, served as controls. Relations between the CSF/plasma proCPU ratio and (a) stroke severity, (b) stroke progression/recurrence, (c) stroke outcome and (d) BBB dysfunction (CSF/serum albumin ratio) were assessed.

Results: Mean (SEM) proCPU levels were elevated in the CSF of stroke patients compared to controls (4.36 (0.23) U/L *vs.* 3.50 (0.23) U/L). Higher median [IQR] CSF/plasma proCPU ratios were found in patients with stroke progression ((6.0 [4.2-6.9])x10^{-3}) and poor outcome ((6.4 [3.9-7.0])x10^{-3}) after 3 months (mRS>3) compared to patients with no progression ((3.9 [2.7-5.4])x10^{-3}) or better outcome ((4.0 [2.8-5.0])x10^{-3}). In stroke patients with a disrupted BBB, proCPU ratios were higher compared to stroke patients with an intact BBB (6.4 [5.8-9.0])x10^{-3} *vs.* (3.7 [2.8-5.0])x10^{-3}).

Conclusions: ProCPU is increased in CSF during hyperacute ischemic stroke and are associated with stroke progression and outcome after 3 months, most likely due to BBB dysfunction in the hyperacute phase of ischemic stroke.

Body text

Introduction

Procarboxypeptidase U (proCPU, TAFI, proCPB2) circulates in plasma as a zymogen and can be activated by plasmin, thrombin and the thrombin-thrombomodulin complex resulting in the formation of active carboxypeptidase U (CPU, TAFIa, CPB2) [1,2]. CPU is a potent antifibrinolytic enzyme and is suggested to modulate inflammation through cleavage of anaphylatoxins C3a and C5a, bradykinin and osteopontin [3,4]. CPU is characterized by its profound thermal instability resulting in conformational changes and loss of function [5]. Due to its antifibrinolytic capacities, the interest in the (pro)CPU system as a target in the treatment of thrombotic events such as acute ischemic stroke (AIS) is rising.

It was reported that proCPU-antigen plasma levels are elevated in the hyperacute phase (<24h after symptom onset) of ischemic stroke compared to controls [6,7], however these studies were compromised by the proCPU-325 polymorphism dependent reactivity of the assays used [8]. Recent studies showed no difference in the proCPU activity levels on admission compared to controls [9,10]. Brouns *et al.* observed a decrease in plasma proCPU levels in the first 72h after stroke onset, reflecting ongoing proCPU activation, that correlated with stroke severity, progression and outcome in non-thrombolyzed AIS patients [9]. Furthermore a decreased efficacy of thrombolytic therapy in patients with increased plasma CPU levels was found and recently, increased CPU+CPUi levels in plasma were linked to poor clinical outcome in AIS patients[10–12].

Baseline levels of proCPU have been detected in cerebrospinal fluid (CSF) of controls [13,14] but so far, no information on proCPU and CPU levels in CSF of AIS patients and its potential relationship to stroke parameters is available. In the current study we investigate proCPU in undiluted CSF of patients with AIS and controls.

Methods

Stroke patients

This study is part of the Middelheim's Interdisciplinary Stroke Study (ZNA Middelheim Hospital, Antwerp, Belgium - 2005-2008), a project on the clinical, biochemical, neuroimaging, neuropsychological, and electrophysiological evaluation of patients with AIS or transient ischemic attack (TIA). On admission, ischemic stroke was confirmed by CT or MRI. ProCPU plasma levels were determined in 136 non-thrombolyzed patients and were previously reported [9]. The current study focuses on 72 of these patients with AIS (n=58) or TIA (n=14) in whom lumbar puncture was performed within 24h after symptom onset. This study was conducted according to the Declaration of Helsinki and approved by the Ethics Committees of ZNA Antwerp and the University of Antwerp.

Controls

Between January and May 2016, diagnostic leftover CSF samples of 32 adult patients with an intact blood-brain barrier (BBB) were collected in the Antwerp University Hospital. These patients underwent lumbar puncture for suspicion of CNS infection, neurological symptoms, memory problems or suspicion of neurodegenerative diseases. Even though, no biochemical or microbiological abnormalities in the CSF were identified. The primary selection criterion for BBB permeability was the age corrected CSF/serum albumin ratio (AR) (n=11), if the AR was not available (n=21), the total protein concentration in CSF (<60 mg/dL) was used to select a control population with an intact BBB [15].

Evaluation of stroke etiology, severity, progression and outcome.

The subtype of AIS was classified according to the TOAST criteria and was dichotomized in cardioembolic and non-cardioembolic etiology[16]. The National Institutes of Health Stroke Scale (NIHSS) was assessed on admission, after 24h, 72h and 7 days by trained physicians to quantify neurological deficit. Patients with a NIHSS-score >7 on admission were considered to have a severe stroke. Stroke progression was evaluated according to the European Progression Stroke Study Criteria (EPSS) [17]. Stroke outcome was assessed by the modified Rankin Scale (mRS) 3 months after the

event, poor outcome was defined as a mRS-score >3 [18]. After 12 months, stroke recurrence was assessed.

Sample collection and biochemical analysis

Lumbar puncture was followed by fractionated sampling in multiple collection tubes. Subsequently, a full work-up of the CSF was performed in the hospital's clinical laboratory. Traumatic taps were excluded if red blood cell count was over 5/µL. CSF samples for proCPU determination were aliquoted and stored at -80°C. Before analysis of proCPU, samples were centrifuged (10 000 *g*, 10 min).

The determination of proCPU in plasma, as well as the AR in this population were previously described [9,19]. In the current study, an *in-house* enzymatic assay with increased selectivity and a shorter turnaround time was used for the determination of proCPU levels in CSF [13,20]. The assay was initially developed for the measurement of proCPU in plasma after dilution (40x) of the samples. As we expected low proCPU levels, CSF samples were not diluted. BBB dysfunction was assessed by the age corrected AR; cutoff values were $7x10^{-3}$ for patients under 40, $8x10^{-3}$ for patients between 40 and 60, $9x10^{-3}$ for patients between 60 and 80 and $10x10^{-3}$ for patients over 80.

Statistical analysis

Statistical computations and data plotting were performed using GraphPad Prism version 7.01. Differences between normally distributed or sufficiently large (n>20) groups, were analyzed with a *t*-test for independent samples; a Mann-Whitney U-test was used for smaller or not normally distributed groups. ProCPU ratios in relation to stroke parameters are shown as median [IQR] unless indicated otherwise. The relations between proCPU levels in CSF and in plasma and between proCPU ratios and parameters for stroke or BBB dysfunction were evaluated by bivariate correlations. Pearson coefficient (*r)* was used for continuous parameters and Spearman's rho (ρ) for ordinal variables.

Results and discussion

The current study is the first to report on proCPU in CSF of stroke patients (n=72) compared to controls (n=32). Patient and stroke characteristics are summarized in **Table 1.** Previously, Lin *et al.* and Matsumoto *et al.* reported on brain-derived, full-length *CPB2* transcripts and shorter, alternatively spliced transcripts without carboxypeptidase activity [21,22]. Baseline levels of proCPU in CSF have been detected in controls and elevated levels were found in patients with traumatic brain injury and bacterial meningitis [13,14]. Antovic *et al*. could not detect proCPU antigen in CSF, most likely due to extensive dilution of the samples before analysis [23].

We modified our existing *in-house* plasma proCPU assay to be applicable in CSF[20]. The modified assay has an excellent linearity (R^2=0.9998) and recovery (94.4-104.8%) after spiking of purified proCPU (0-15 U/L) in normal pooled CSF. The limit of detection is 0.2 U/L as determined by the ICH harmonized tripartite guideline.

In the hyperacute phase (<24h after symptom onset) of ischemic stroke, mean (SEM) proCPU levels in CSF were higher compared to controls (4.36 (0.23) U/L *vs.* 3.50 (0.23) U/L; *t*-test, *P*<0.05) (**Fig. 1**). Plasma concentrations of proCPU in the (sub)acute phase of AIS were previously determined by our research group [9]. On admission in the hospital, mean (SEM) proCPU plasma levels of the current study corpus of 72 patients were 959 (19) U/L. The mean (SD) time interval between blood sampling and lumbar puncture was 0.7 (1.0)h, the interval between symptom onset and lumbar puncture was 9.0 (6.4)h. None of these intervals influenced proCPU levels in CSF or plasma (*r*=-0.085 and 0.076 respectively*; P*>0.05). ProCPU levels in CSF of non-thrombolyzed stroke patients are 200-225x lower compared to plasma levels and we observed no correlation between plasma and CSF levels in the stroke population (*r*=0.170*; P*>0.05; data not shown).

BBB dysfunction

During AIS, the BBB is frequently compromised which is associated with acute complications (e.g. cerebral edema). The long-term consequences of BBB dysfunction during AIS are diverse and the key mechanisms are not completely elucidated yet [24].

Brouns *et al.* indicated that the CSF/serum albumin ratio (AR) is a reliable biomarker for BBB dysfunction in hyperacute stroke and that the extent of BBB disruption relates to stroke severity, unfavorable stroke evolution and poor long-term outcome[19]. In the current study cohort, that is part of the same population as the one investigated by Brouns and coworkers, the AR was determined for 67 patients and we evaluated the correlation between the AR and the CSF/plasma proCPU ratio. We found an apparent correlation between the proCPU ratio and the AR (**Fig. 2A**; r=0.65; P<0.0001). As assessed by the age corrected AR, proCPU ratios were higher in patients with BBB dysfunction (n=11) compared to patients with an intact BBB (n=56) (**Fig. 2B**; (6.4 [5.8-9.0])x10^{-3} *vs.* (3.7 [2.8-5.0])x10^{-3}; Mann-Whitney U, P<0.001).

Stroke parameters

As displayed in **Fig. 3A**, the CSF/plasma proCPU ratio was increased in patients with progressive stroke (6.0 [4.2-6.9])x10^{-3}) compared to non-progressive stroke ((3.9 [2.7-5.4])x10^{-3}; Mann-Whitney U, P<0.01). Increased proCPU ratios were also observed in patients with poor outcome (**Fig. 3B**) assessed by the mRS-score and mortality ((4.0 [2.8-5.0])x10^{-3} for mRS 0-3 *vs.* (6.4 [3.9-7.0])x10^{-3} for mRS 4-6 and (4.0 [2.8-5.2])x10^{-3} in surviving patients *vs.* (6.4 [4.8-7.5])x10^{-3} in deceased patients; Mann-Whitney U, P<0.01 and P<0.05 respectively). We observed statistically significant correlations of the proCPU ratio with stroke progression (ρ=0.30, P<0.01) and stroke outcome that was assessed by the mRS-score and mortality at month 3 (ρ=0.30, P<0.05 and ρ=0.31, P<0.01 respectively).

Mean (SEM) proCPU ratios in patients with cardioembolic stroke did not differ significantly from those in patients with non-cardioembolic stroke ((4.63 (0.31))x10^{-3} *vs.* (4.58 (0.38))x10^{-3}; t-test, P>0.05). ProCPU ratios did neither differ significantly between mild and severe strokes ((4.66 (0.31))x10^{-3} for

NIHSS≤7 *vs*. (4.53 (0.38))x10^{-3} for NIHSS>7; *t*-test, *P*>0.05) nor between TIA and AIS (median [IQR]; (3.9 [2.7-5.8])x10^{-3} *vs*. (4.3 [3.0-6.2])x10^{-3}); Mann-Whitney U, *P*>0.05). Patients with stroke recurrence did not present with higher proCPU ratios compared to patients without recurrence ((4.8 [3.7-6.7])x10^{-3} *vs*. (4.1 [2.9-6.1])x10^{-3}; Mann-Whitney U, *P*>0.05). There were no direct correlations between proCPU ratios and stroke severity (NIHSS, TIA/AIS) or recurrence (*r*=0.23 and *ρ*=0.06 and 0.11 respectively, *P*>0.05).

The increase of proCPU in CSF of patients with stroke progression and unfavorable outcome is most likely secondary to BBB dysfunction. It was shown that patients with BBB dysfunction are prone to stroke progression and poor outcome[19]. Additionally our data show a strong correlation of the proCPU ratio with BBB dysfunction. Furthermore, after excluding patients with BBB dysfunction, the increase in proCPU levels was no longer observed in stroke patients compared to controls (Mean (SEM); 3.50 (0.23)U/L *vs.* 3.98 (0.22)U/L; *t*-test P>0.05). It is also doubtful that a mean increase of proCPU in CSF of approximately 1 U/L could have a significant influence on pathophysiology, although higher local concentrations cannot be excluded. The low circulating levels of proCPU in CSF - even after complete conversion into active CPU - will probably be too low to exert its antifibrinolytic effect in the brain, especially considering the fact that CPU attenuates fibrinolysis through a threshold dependent mechanism that is dependent on the tPA concentration [25]. As the major components of the plasminogen activating system (including tPA) are strongly expressed in the central nervous system [26], a high CPU threshold value is to be expected. From the moment the CPU activity falls under the threshold level, fibrinolysis accelerates exponentially and becomes uncontrollable by the residual CPU.

The fibrinolytic pathway and its effector enzyme, plasmin are also closely linked to the activation and regulation of the complement system. The proCPU system modulates inflammation through cleavage of anaphylatoxins such as C3a and C5a [27]. The complement system plays a distinct role in cerebrovascular inflammation after ischemic stroke and plasma levels of C3a and C5a are increased in

the (sub)acute phase of ischemic stroke [28,29]. Furthermore, an increase in C3 and C3a plasma levels in patients with cardioembolic stroke was linked with unfavorable long-term outcome[30]. It is therefore arguably possible that increased proCPU levels in CSF may influence acute neuro-inflammation during AIS.

A possible limitation of the study is that the stroke and control population were not age and sex matched. Correction of the proCPU concentrations was not performed as age- and sex-dependent reference intervals for proCPU have not been established. This problem is partly solved by measuring CSF/plasma ratios for the stroke population. Matched plasma samples however were not available for the controls as we used clinical leftover CSF samples. In the future it might also be interesting to look for active CPU in CSF, but this would require a special sampling procedure using PPACK and aprotinin [31].

Conclusion

In conclusion, this study is the first to survey proCPU levels in CSF of stroke patients. During hyperacute ischemic stroke, proCPU is increased in CSF compared to controls. CSF/plasma proCPU ratios correlate with stroke progression and outcome. These findings are most likely secondary to BBB dysfunction in the hyperacute phase of ischemic stroke.

Addendum

R. Brouns, S. Engelborghs, P.P. De Deyn, J.C. Mertens, D. Leenaerts and D. Hendriks: Study concept and design. R. Brouns: CSF sampling stroke patients. M. Ieven and J.C. Mertens: selection and recruitment of control population. J.C. Mertens and D. Leenaerts: Sample processing and measurements. P. van der Veken: Synthesis of selective substrate. J.C. Mertens: statistical analysis and interpretation of data. J.C. Mertens: drafting of the manuscript. D. Leenaerts, R. Brouns, S. Engelborghs, M. Ieven, P.P. De Deyn, A.M. Lambeir, D. Hendriks: critical revision of the manuscript for important intellectual content.

Acknowledgements

J.C. Mertens and D. Leenaerts are research assistants of the Fund for Scientific Research Flanders (FWO Vlaanderen). R. Brouns is a senior clinical investigator of the FWO Vlaanderen. This research was also supported by: the Institute Born-Bunge; the agreement between the Institute Born-Bunge and the University of Antwerp; the Interuniversity Attraction Poles (IAP) program P6/43 of the Belgian Federal Science Policy Office, Belgium; and the Medical Research Foundation Antwerp. The authors would like to thank Y. Sim and N. De Roeck for their excellent technical assistance and S. Vandamme, MD and I. Mermans for their help with the recruitment of the control samples. We dedicate this paper to our colleague, Prof Sandra Apers, who passed away much too early on February 5th, 2017.

References

1 Hendriks D, Scharpé S, van Sande M, Lommaert MP. Characterisation of a carboxypeptidase in human serum distinct from carboxypeptidase N. *J Clin Chem Clin Biochem* 1989; **27**: 277–85.

2 Hendriks D, Scharpé S, van Sande M, Lommaert MP. A labile enzyme in fresh human serum interferes with the assay of carboxypeptidase N. *Clin Chem* 1989; **35**: 177.

3 Leurs J, Hendriks D. Carboxypeptidase U (TAFIa): A metallocarboxypeptidase with a distinct role in haemostasis and a possible risk factor for thrombotic disease. *Thromb Haemost* 2005; **94**: 471–87.

4 Campbell WD, Lazoura E, Okada N, Okada H. Inactivation of C3a and C5a octapeptides by carboxypeptidase R and carboxypeptidase N. *Microbiol Immunol* 2002; **46**: 131–4.

5 Boffa MB, Bell R, Stevens WK, Nesheim ME. Roles of thermal instability and proteolytic cleavage in regulation of activated thrombin-activable fibrinolysis inhibitor. *J Biol Chem* 2000; **275**: 12868–78.

6 Montaner J, Ribó M, Monasterio J, Molina C a, Alvarez-Sabín J. Thrombin-activable fibrinolysis inhibitor levels in the acute phase of ischemic stroke. *Stroke* 2003; **34**: 1038–40.

7 Rooth E, Wallen H, Antovic A, von Arbin M, Kaponides G, Wahlgren N, Blombäck M, Antovic J. Thrombin activatable fibrinolysis inhibitor and its relationship to fibrinolysis and inflammation during the acute and convalescent phase of ischemic stroke. *Blood Coagul Fibrinolysis* 2007; **18**: 365–70.

8 Heylen E, Willemse JL, Hendriks DF. Comparative study of commercially available procarboxypeptidase U (thrombin-activatable fibrinolysis inhibitor) assays. *J Thromb Haemost* 2011; **9**: 1407–9.

9 Brouns R, Heylen E, Willemse JL, Sheorajpanday R, De Surgeloose D, Verkerk R, De Deyn PP,

Hendriks DF. The decrease in procarboxypeptidase U (TAFI) concentration in acute ischemic stroke correlates with stroke severity, evolution and outcome. *J Thromb Haemost* 2010; **8**: 75–80.

10 Alessi M-C, Gaudin C, Grosjean P, Martin V, Timsit S, Mahagne M-H, Larrue V, Sibon I, Zuber M, Brouns R, Montaner J, Castellanos M, Donazzolo Y, Cho T-H, Suissa L, Mechtouff L, Derex L, Suchon P, Mezzapesa A, Nighoghossian N. Changes in Activated Thrombin-Activatable Fibrinolysis Inhibitor Levels Following Thrombolytic Therapy in Ischemic Stroke Patients Correlate with Clinical Outcome. *Cerebrovasc Dis* 2016; **42**: 404–14.

11 Brouns R, Heylen E, Sheorajpanday R, Willemse JL, Kunnen J, De Surgeloose D, Hendriks DF, De Deyn PP. Carboxypeptidase U (TAFIa) decreases the efficacy of thrombolytic therapy in ischemic stroke patients. *Clin Neurol Neurosurg* 2009; **111**: 165–70.

12 de Bruijne ELE, Gils A, Guimarães AHC, Dippel DWJ, Deckers JW, Van Den Meiracker AH, Poldermans D, Rijken DC, Declerck PJ, De Maat MPM, Leebeek FWG. The role of thrombin activatable fibrinolysis inhibitor in arterial thrombosis at a young age: The ATTAC study. *J Thromb Haemost* 2009; **7**: 919–27.

13 Leenaerts D, Feyen B, Sener S, Maas A, Jorens P, Sim Y, Ieven M, Lambeir A-M, Hendriks D. Identification of functional procarboxypeptidase U (proCPU, proCPB2, TAFI) in cerebrospinal fluid and its enhancement in the acute phase of brain injury. *J Thromb Haemost* 2015; **13 Suppl.2**: 342.

14 Mook-Kanamori BB, Valls Serón M, Geldhoff M, Havik SR, van der Ende A, Baas F, van der Poll T, Meijers JCM, P. Morgan B, Brouwer MC, van de Beek D. Thrombin-activatable fibrinolysis inhibitor influences disease severity in humans and mice with pneumococcal meningitis. *J Thromb Haemost* 2015; **13**: 2076–86.

15 Burtis CA, Edward RA, Bruns DE. Tietz Textbook of Clinical Chemistry and Molecular Diagnostics.

4th ed. Burtis CA, Ashwood ER, Burns DE, editors. Philadelphia, Pa: Saunders; 2005.

16 Adams HP, Bendixen BH, Kappelle LJ, Biller J, Love BB, Gordon DL, Marsh EE. Classification of Subtype of Acute Ischemic Stroke. *Stroke* 1993; **23**: 35–41.

17 Birschel P, Ellul J, Barer D. Progressing stroke: Towards an internationally agreed definition. *Cerebrovasc Dis* 2004; **17**: 242–52.

18 van Swieten JC, Koudstaal PJ, Visser MC, Schouten HJ, van Gijn J. Interobserver agreement for the assessment of handicap in stroke patients. *Stroke* 1988; **19**: 604–7.

19 Brouns R, Wauters A, De Surgeloose D, Mariën P, De Deyn PP. Biochemical markers for blood-brain barrier dysfunction in acute ischemic stroke correlate with evolution and outcome. *Eur Neurol* 2011; **65**: 23–31.

20 Heylen E, Van Goethem S, Willemse J, Olsson T, Augustyns K, Hendriks D. Development of a sensitive and selective assay for the determination of procarboxypeptidase U (thrombin-activatable fibrinolysis inhibitor) in plasma. *Anal Biochem* 2010; **396**: 152–4.

21 Matsumoto A, Motozaki K, Seki T, Sasaki R, Kawabe T. Expression of human brain carboxypeptidase B, a possible cleaving enzyme for β-amyloid precursor protein, in peripheral fluids. *Neurosci Res* 2001; **39**: 313–7.

22 Lin JHH, Novakovic D, Rizzo CM, Zagorac B, Garand M, Filipieva A, Koschinsky ML, Boffa MB. The mRNA encoding TAFI is alternatively spliced in different cell types and produces intracellular forms of the protein lacking TAFIa activity. *Thromb Haemost* 2013; **109**: 1033–44.

23 Antovic J, Hannerz J, Nekludov M, Margareta B. Thrombin activatable fibrinolysis inhibitor antigen could not be detected in cerebrospinal fluid. *Thromb Haemost* 2005; **93**: 178–9.

24 Schoknecht K, Shalev H. Blood-brain barrier dysfunction in brain diseases: clinical experience. *Epilepsia* 2012; **53 Suppl 6**: 7–13.

25 Leurs J, Nerme V, Sim Y, Hendriks D. Carboxypeptidase U (TAFIa) prevents lysis from proceeding into the propagation phase through a threshold-dependent mechanism. *J Thromb Haemost* 2004; **2**: 416–23.

26 Medcalf RL. Fibrinolysis, inflammation, and regulation of the plasminogen activating system. *J Thromb Haemost* 2007; **5**: 132–42.

27 Leung LL, Morser J. Plasmin as a complement C5 convertase. *EBioMedicine* 2016; **5**: 20–1.

28 Orsini F, De Blasio D, Zangari R, Zanier ER, De Simoni M-G. Versatility of the complement system in neuroinflammation, neurodegeneration and brain homeostasis. *Front Cell Neurosci* 2014; **8**: 380.

29 Mocco J, Wilson DA, Komotar RJ, Sughrue ME, Coates K, Sacco RL, Elkind MS V, Connolly ES. Alterations in plasma complement levels after human ischemic stroke. *Neurosurgery* 2006; **59**: 28–32.

30 Stokowska A, Olsson S, Holmegaard L, Jood K, Blomstrand C, Jern C, Pekna M. Cardioembolic and Small Vessel Disease Stroke Show Differences in Associations between Systemic C3 Levels and Outcome. *PLoS One* 2013; **8**.

31 Willemse JL, Brouns R, Heylen E, De Deyn PP, Hendriks DF. Carboxypeptidase U (TAFIa) activity is induced in vivo in ischemic stroke patients receiving thrombolytic therapy. *J Thromb Haemost* 2008; **6**: 200–2.

Tables and figures

Table 1

Table 1: Baseline characteristics and stroke characteristics of patients and controls

Characteristic	Stroke patients (n = 72)	Controls (n = 32)
Age, years; mean (SD)	71 (14)	54 (17)
Male gender, n (%)	43 (59.7)	15 (46.9)
TIA *vs.* ischemic stroke, n (%)	14 (19.4) *vs.* 58 (80.6)	-
NIHSS-score*, mean (SD)	8 (10)	-
Severe stroke*, n (%)	26 (36.1)	
Progressive stroke†, n (%)	14 (19.4)	-
mRS (0-3 *vs.* 4-6) at month 3, n (%)	53 (73.6) *vs.* 19 (26.4)	-
Mortality at month 3, n (%)	13 (18.1)	-
Stroke recurrence at 12 months, n (%)	9 (12.5)	-
Stroke Etiology (TOAST), n (%)		
Cardioembolic	31 (43.1)	-
Lacunar	20 (27.8)	-
Atherothrombotic	12 (16.7)	-
Other determined origin	3 (4.2)	-
Undetermined	6 (8.3)	-

TIA: Transient Ischemic Attack, NIHSS: National Institutes of Health Stroke Scale, mRS: modified Rankin Scale, TOAST: Trial of ORG 10172 in Acute Stroke Treatment.
*NIHSS-score assessed on admission in the hospital; severe stroke: NIHSS-score on admission >7.
†Progressive stroke was determined according to the European Progression Stroke Study Criteria.

Figure 1

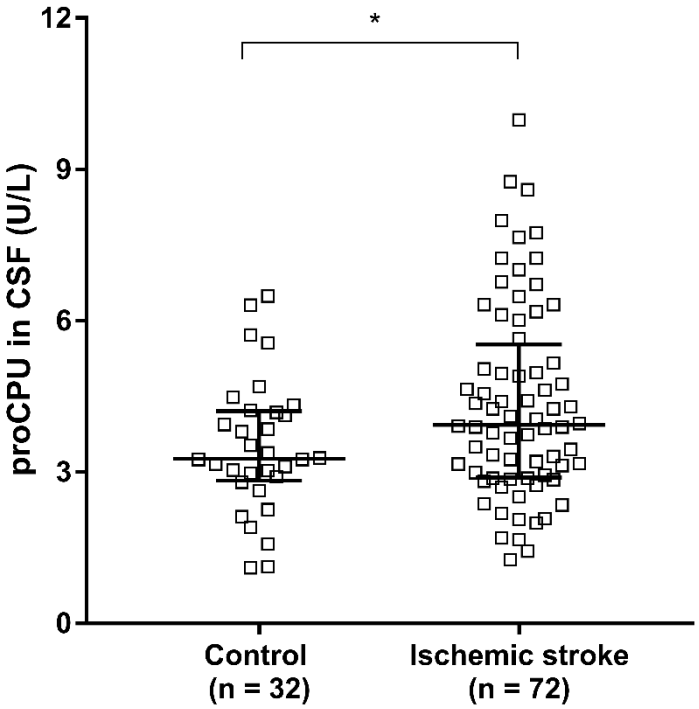

Fig. 1: ProCPU levels in CSF of controls and non-thrombolyzed acute ischemic stroke patients. Dot plot of procarboxypeptidase U (proCPU) levels in CSF of controls (n = 32) compared to acute ischemic stroke patients (n = 72). The horizontal lines indicate medians, whiskers represent interquartile range (IQR). ProCPU in CSF of stroke patients was significantly higher compared to controls (*t-test for independent samples; $P < 0.05$).

Figure 2

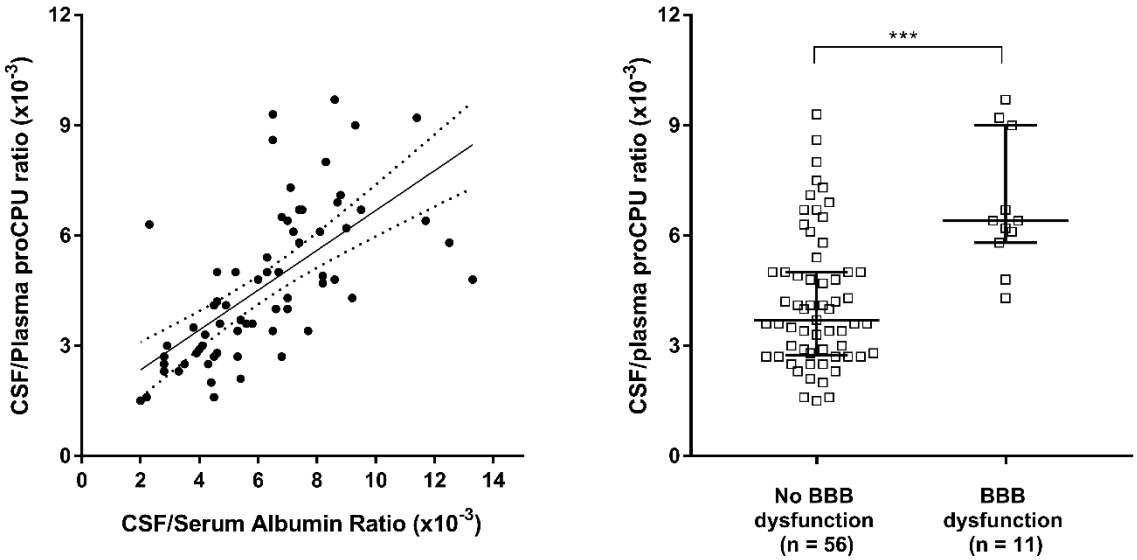

Fig. 2: ProCPU ratio in relation to BBB dysfunction. Panel A. Dot plot of the correlation between the CSF/plasma proCPU ratio and the CSF/serum albumin ratio (AR). The solid line represents the linear regression curve, dashed lines represent the 95% Confidence Interval. (Pearson *r*: 0.65, $P < 0.0001$). **Panel B.** Dot plot of the CSF/plasma proCPU ratio of acute ischemic stroke patients with an intact BBB according to the age corrected AR compared to stroke patients with BBB dysfunction. Increased proCPU ratios were associated with BBB dysfunction (***Mann-Whitney U, $P < 0.001$). Long horizontal lines indicate medians, whiskers represent Interquartile Range (IQR).

Figure 3

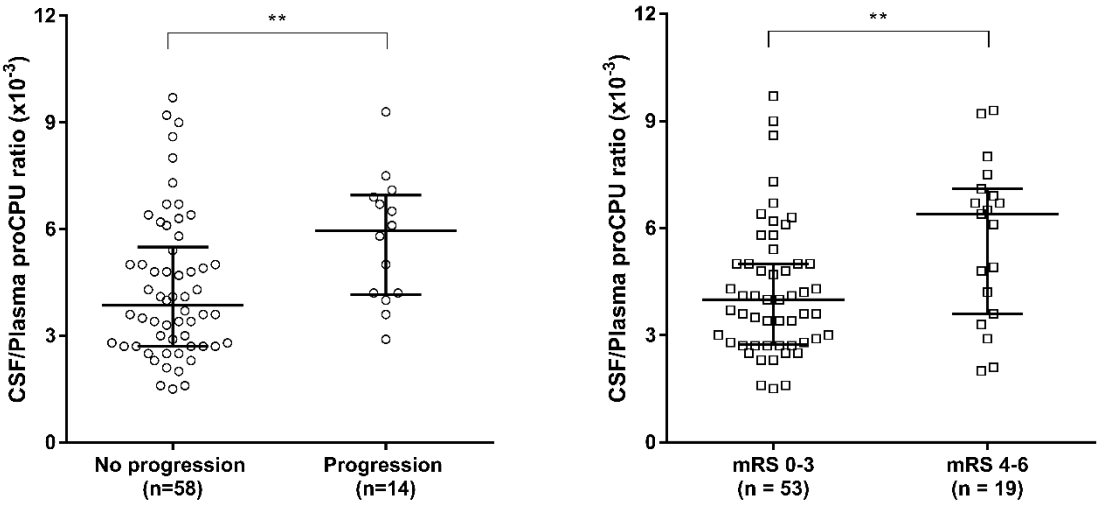

Fig. 3: ProCPU levels in relation to stroke progression and stroke outcome. **Panel A.** Dot plot of the CSF/plasma proCPU ratio of acute ischemic stroke patients with progressive stroke compared to patients without stroke progression based on the EPSS criteria. **Panel B.** Dot plot of the CSF/plasma proCPU ratio of acute ischemic stroke patients with a moderate to good outcome (modified Rankin Scale, mRS-score 0-3) compared to poor outcome (mRS-score 4-6). Stroke patients with progressive stroke and poor outcome had higher proCPU ratios compared to patients without progression and relatively better outcome (**Mann-Whitney U; $P < 0.01$). Long horizontal lines indicate medians, whiskers represent Interquartile Range (IQR).