

Plasma C3 and C3a levels in cryptogenic and large vessel disease stroke: associations with outcome

Anna Stokowska^a, Sandra Olsson^a, Lukas Holmegaard^a, Katarina Jood^a, Christian Blomstrand^a, Christina Jern^{a,*}, Marcela Pekna^{a,*}

^aInstitute of Neuroscience and Physiology, Department of Clinical Neuroscience and Rehabilitation, The Sahlgrenska Academy at University of Gothenburg, Gothenburg, Sweden.

* Corresponding authors:

Dr. Marcela Pekna, MD, PhD

Institute of Neuroscience and Physiology, Dept. of Clinical Neuroscience and Rehabilitation
The Sahlgrenska Academy at University of Gothenburg

Medicinaregatan 9a, Box 440, SE-405 30 Göteborg, Sweden

Tel: +46-31-786 3581, FAX: +46-31-416 108

E-mail: Marcela.Pekna@neuro.gu.se

Prof. Christina Jern, MD, PhD

Institute of Neuroscience and Physiology, Dept. of Clinical Neuroscience and Rehabilitation
The Sahlgrenska Academy at University of Gothenburg

Per Dubbsgatan 14, SE-413 45 Göteborg, Sweden

Tel: +46-31-343 5720, FAX: +46-31-842 160

E-mail: Christina.Jern@neuro.gu.se

This is an electronic version of an Article published in *Cerebrovascular Disease* © 2011 S. Karger AG, Basel.

<http://content.karger.com/produktedb/produkte.asp?DOI=000328238&typ=pdf>

Published in *Cerebrovascular Disease*. 32:114-22.

ABSTRACT

Background and Purpose

Inflammation seems to be a key player in the pathophysiology of stroke. In this study, we compared plasma C3 and C3a levels in cryptogenic and large vessel disease (LVD) subtypes of ischemic stroke and control subjects and evaluated their association to outcome at three months and two years.

Methods

C3 and C3a in plasma of 79 cryptogenic and 73 LVD stroke patients, sampled within 10 days and at three months after stroke, and age- and sex-matched control subjects from the Sahlgrenska Academy Study on Ischemic Stroke was measured by ELISA. Functional outcome was assessed with modified Rankin Scale.

Results

Plasma C3 was increased in both stroke groups at both time points. Systemic elevation of C3a was limited to the acute phase in the cryptogenic stroke group, whereas plasma C3a levels in the LVD group were elevated also at three-month follow-up. In the LVD group, three month follow-up plasma C3 levels in the upper third were associated with unfavourable outcome after three months independently of age and sex: OR 5.56; 95% CI 1.03-29.93; $P=0.045$; as well as after two years: OR 4.75; 95% CI 1.11-20.30; $P=0.036$. In the cryptogenic stroke group, high plasma C3a levels in the acute phase were associated with unfavourable outcome after three months: OR 3.75; 95% CI 1.01-13.96; $P=0.049$ in univariate analysis but not after adjustment for age and sex ($P=0.050$).

Conclusions

Plasma C3 and C3a levels are elevated in cryptogenic and LVD stroke and the predictive value of these markers may depend on stroke subtype. Further studies on the role of the complement system in ischemic stroke outcome, based on larger patient populations and controlling for the effect of infections, are clearly warranted.

INTRODUCTION

Inflammation seems to play a key role in the pathophysiology of stroke [1, 2] as well as in the development and destabilization of atherosclerotic plaques [3, 4], and progression of symptomatic intracranial large vessel disease (LVD) is associated with a proinflammatory state [5]. Plasma levels of several inflammatory markers have recently been shown to be altered during the first 7 days after ischemic stroke and to be independently related to clinical outcome scores on day 90 [6]. The complement system is a major inflammatory mediator that appears to play a dual role in experimental stroke. Complement plays a positive role in normal CNS maintenance and genetic deficiency of the third complement component (C3) was associated with larger cerebral infarcts after permanent focal brain ischemia [7]. Anaphylatoxic peptides C3a and C5a, generated in the process of complement activation, can be protective against excitotoxicity-induced neuronal death and can ultimately contribute to healing [8,9]. On the other hand, inhibition of complement activation reduced infarct volume and neurological impairments after transient cerebral ischemia in animal models [10-13]. In humans, the role of complement in ischemic stroke is less clear. Complement is activated locally in the human brain in the course of ischemic stroke as the deposits of both early complement components (C1q, C3c and C4d) and terminal complex (C5b-9) were found in the infarcts [14, 15]. Plasma levels of C5b-9, C4d, C3 and C3a were shown to be elevated in the acute phase of ischemic stroke [16-18] and the levels of soluble C5b-9 positively correlated with the clinical severity of stroke, the degree of neurological deficit and functional disability 6 days after admission [16]. However, these studies involved only small patient groups that did not allow for separate analysis of complement system response in individual stroke subtypes.

In the present study, we compared plasma levels of C3 and its activation product C3a in control subjects and in two etiologically different ischemic stroke subtypes, namely LVD and cryptogenic stroke. We investigated association of plasma C3 and C3a levels to stroke, correlations to C-reactive protein (hsCRP), and functional outcome at three months and two years post admission.

SUBJECTS AND METHODS

Study population

The study population comprises participants in the Sahlgrenska Academy Study on Ischemic Stroke (SAHLIS) and population-based controls free from stroke and clinical atherothrombotic disease [19]. In short, patients who presented with first-ever or recurrent acute ischemic stroke before reaching the age of 70 years were consecutively recruited (n=600). Healthy community Caucasian controls (n=600) were randomly selected as described. All patients underwent neuroimaging and were examined by a physician, both at admission and at three-month follow up. The patients were classified into stroke subtypes according to the Trial of Org 10172 in Acute Stroke Treatment (TOAST) criteria. The distribution of stroke etiologic subtypes in the population was as follows: LVD (n=73, 12%), SVD (n=124, 21%); CE (n=98, 16%); cryptogenic stroke (n=162, 27%); other determined stroke (n=51, 9%); and undetermined stroke (n=92, 15%). Cryptogenic stroke was defined when no cause was identified despite an extensive evaluation. Undetermined stroke included cases for which more than one cause was identified or when the evaluation was cursory. Maximum stroke severity within the first 10 days after the stroke was scored using the Scandinavian Stroke Scale (SSS). SSS is a validated measure of neurological impairment. However, it should be noted that it differs in the direction of measurement compared to the National Institutes of Health Stroke Scale (NIHSS),

e.g. no impairment is 58 of 58 in the SSS and 0 of 42 in the NIHSS. Functional outcome after three months and two years was assessed with the modified Rankin Scale (mRS).

In the present study, we investigated ischemic stroke due to atherosclerosis, i.e. LVD, and stroke due to an unknown cause, i.e. cryptogenic stroke. To this end, all patients classified as having stroke due to LVD (n=73) and a similar number of patients with cryptogenic stroke (n=79, i.e. half of the patients classified as having this subtype) were included. Because the cryptogenic group has a relatively favourable functional outcome, all cryptogenic stroke patients with a mRS score >2 were selected (n=31) to minimize the effect of uneven distribution of outcome categories in this subgroup and thus improve the statistical power in the outcome regression analysis. The other 48 cryptogenic stroke patients were selected so that they represented an even distribution of the mRS scores 0, 1 and 2, i.e. a similar number of patients with each score was randomly selected. For each of the two groups half the number of controls (selected so as to have the same mean age and sex distribution as the cases) was analyzed.

The study was approved by the Ethical Committee of the University of Gothenburg. All participants provided written informed consent prior to enrolment. For those participants who were unable to communicate, consent was obtained from the next-of-kin.

Blood sampling

In patients, blood sampling was performed within 10 days of the stroke event (median day of sampling was 4.5 and 5.0 after the stroke event, for the cryptogenic and LVD group respectively) and at the three-month follow up. In controls, blood sampling was performed once. Venous blood was collected in ethylenediaminetetraacetic

acid (EDTA-Vacurette tubes, Greiner) between 8:30 and 10:30 AM after an overnight fast, centrifuged and supernatants were stored at -80°C. Serum levels of high sensitivity CRP (hsCRP) were analysed as reported previously [20].

C3 and C3a measurements

Plasma C3 was measured by sandwich ELISA. Wells of microtitre plates were coated with polyclonal rabbit anti-C3c antibody (DAKO, Glostrup, Denmark) in phosphate buffered saline (PBS) at 4°C overnight. The plates were saturated with dilution buffer which is PBS containing 0.05% (v/v) Tween 20 (Sigma, St. Louis, MO, USA) and 1% bovine serum albumin (Sigma) for 30 min at room temperature (RT). Sample or human C3 (Sigma) used as a standard was incubated for 60 min at RT. Horseradish peroxidase-conjugated polyclonal anti-C3c antibody (DAKO) was incubated for 60 min at RT. After each incubation step, the plates were washed with PBS containing 0.05% (v/v) Tween 20. The enzyme reaction was started by addition of colour reagent: 20 mg of 1,2-phenyldiamine-dihydrochloride (Sigma), and 10 μ l H₂O₂ in 75 ml 0.1 M citrate/phosphate buffer, pH 5. The reaction was stopped by addition of 1 M H₂SO₄ after 3 min. The staining was quantified spectrophotometrically at 490 nm. Plasma samples were analyzed for C3a by ELISA kit (Quidel Corporation, San Diego, CA, USA) according to manufacturer's recommendations.

Statistical analysis

Due to predominantly non-Gaussian distribution of C3 and C3a values, non-parametric tests were employed for the statistical analysis. Differences in characteristics between cases and controls were examined with the χ^2 test for proportions for categorical variables and the Mann-Whitney *U* test for continuous variables. Plasma levels of C3 and C3a are presented as medians and inter-quartile ranges. Time-point differences of C3 and

C3a levels were compared using Wilcoxon Signed Rank test. Correlations between plasma levels of C3, C3a and serum levels of hsCRP were estimated by Spearman's rank correlation coefficient, with a two-tailed significance test. For the regression models of the association with stroke, values for the C3 and C3a levels were standardised using mean and standard deviation of the respective control population. Furthermore, the values of complement proteins levels were divided into tertiles.

The association between complement levels and case/control status was investigated with binary logistic regression adjusted for the established risk factors, age, sex, hypertension, smoking status, diabetes mellitus (Model 1). For studying association of complement levels and functional outcome at three months and two years (after stroke favourable, mRS 0-2 versus unfavourable, mRS 3-6) a regression model adjusted for age and sex (Model 2) was used. ORs and 95% CIs were calculated separately for the two ischemic stroke subtypes, where patient groups were compared to their respective control group.

The cryptogenic and LVD group samples were analysed on two separate occasions more than a year apart. Therefore, to avoid erroneous conclusions resulting from possible change in reagents and plasma sample stability between the two occasions, direct comparisons were performed only between patients and their simultaneously analysed controls but not between the subtype groups.

Statistical analysis was performed in R statistical package 2.10.1. Adjustment for multiple testing was not conducted as our study was considered to be hypothesis generating.

Missing values

The numbers of individuals with missing values were as follows: smoking status 2, hypertension 3, SSS score 4, hyperlipidemia 10, hsCRP 14 (acute 5, follow-up 9), C3 17 (acute 6, follow-up 11), C3a 17 (acute 6, follow-up 11) and mRS 7 (three months 5, two years 2). In the regression analysis, for the categorical variables with missing values, respective dummy variables were created and included in the model. Dummy was coded 1 when the value of a parameter was missing and 0 when the value was present. These variables were not significant at any point ($P>0.97$), indicating that missing values did not affect the results of regression analysis.

The manuscript was prepared according to the STROBE guidelines [21].

RESULTS

Demographic and clinical characteristics for the patient and control groups whose samples were used for the analysis of plasma levels of C3 and C3a are summarized in Table 1. Hypertension and smoking were more common in cases than in controls; diabetes was more common in patients with LVD stroke than in the corresponding control subjects. The LVD group also displayed significantly higher levels of hsCRP compared to controls.

Plasma C3 and C3a levels after ischemic stroke

In both cryptogenic and LVD stroke groups, plasma C3 levels at both time points were elevated compared to the control groups. C3 levels in the acute phase were significantly higher compared to the follow-up samples in the LVD group ($P<0.01$), whereas in the cryptogenic stroke group C3 levels at three month follow-up did not differ from levels in the acute phase (Fig. 1A). In the acute phase, plasma levels of C3a were higher in patients than in the control groups in both

stroke subtypes ($P<0.001$). At three months follow-up, C3a levels declined in both stroke subtype groups ($P<0.001$), however in the LVD group, C3a were still elevated above the control levels ($P<0.05$; Fig. 1B).

C3 levels were positively correlated to hsCRP at both time points in the cryptogenic group. In this group, plasma C3a levels in the acute phase correlated negatively to C3, and C3a levels at three-month follow-up were positively correlated to hsCRP. In the LVD group, acute plasma C3a levels were positively correlated with C3 and hsCRP levels; plasma C3a levels at three-month follow-up correlated with hsCRP (Table 2).

There was no significant difference in acute phase SSS between the LVD and the cryptogenic stroke group (Table 1). Furthermore, there were no significant correlations between SSS and the C3 or C3a levels, with the exception for an inverse correlation between this score and the C3 level at follow-up in the LVD group (Table 2). In contrast, in both the cryptogenic and LVD group, we observed an inverse correlation between acute phase SSS and acute hsCRP levels ($\rho = -0.41$, $P<0.001$, and $\rho = -0.36$, $P=0.002$, respectively) but not follow-up hsCRP levels.

Association of plasma C3 and C3a levels with stroke

Detailed results of binary logistic regression analysis of the case/control status are presented in Table 3 and values of cut-offs for C3 and C3a tertiles are listed in Table 4. Acute phase plasma C3 concentration in the upper and middle third was associated with stroke in cryptogenic group both in the univariate and multivariate analysis (adjusted for age, sex, smoking status, hypertension and diabetes). In the LVD group, acute phase plasma C3 levels in the upper third were associated with stroke in both models.

Follow-up plasma C3 levels in the upper third were associated with stroke in the cryptogenic group only in the univariate model, whereas plasma C3 levels at this time point in the LVD group were associated with stroke in both regression models.

Higher acute C3a levels were also associated with stroke in both subtypes under the unadjusted as well as under the multiple-adjusted model. At three months after stroke, elevated C3a plasma concentration was associated with case/control status only in the LVD group. This association was independent of traditional risk factors.

Association of plasma C3 and C3a levels with functional outcome

Among the investigated cryptogenic stroke patients, 28 (37%) and 22 (30%) had an unfavourable outcome (mRS 3-6) at three months and at two years after the stroke event, respectively. In the LVD stroke group, 17 patients (27%) and 21 patients (32%) had an unfavourable outcome at three-month and two-year follow-up, respectively. The acute phase plasma C3a levels in the upper third were found to be associated with an increased risk of unfavourable outcome at three months in the cryptogenic stroke group in the univariate analysis. However, this association was lost after adjustment for age and sex. In the LVD group, three month follow-up plasma C3 levels in the upper third were associated with unfavourable outcome after three months as well as after two years both in the univariate analysis and after adjustment for age and sex (Table 5).

DISCUSSION

Published reports on systemic complement activation after ischemic stroke have been based on studies involving small patients groups that did not allow for separate analysis of complement system response in individual etiologic subtypes of ischemic

stroke [18, 22]. Furthermore, there are only a limited number of reports on associations between complement levels and clinical outcome. Here, we have found that both in cryptogenic and LVD stroke, plasma levels of both C3 and C3a were elevated in the acute phase and were associated with ischemic stroke also after adjustment for the influence of traditional risk factors (age, sex, smoking, hypertension and diabetes). These results confirm previous findings showing elevated plasma C3a levels during the first 7 days after ischemic stroke [18] and suggest that systemic complement activation in the early post-stroke phase is an important contributor to the inflammatory response after ischemic stroke. This notion is supported by a previous study that found increased plasma levels of C5b-9 during the first 12 days after ischemic stroke [22]. In both stroke subtypes, C3 levels remained elevated also three months after stroke. In the LVD group, high plasma C3 levels at this time point showed an association with patient status independently of traditional risk factors. This finding points to the potential role of C3 in the pathophysiological mechanisms of ischemic stroke.

We observed differences between the stroke subtypes in terms of complement response. Plasma C3a and C3 levels showed a positive correlation only in the LVD group and only in the acute phase. Plasma C3a levels remained elevated at the follow-up only in the LVD group and showed a significant association with the case/control status. As atherosclerosis has been shown to be strongly associated with complement activation [23], it is conceivable that the elevation of plasma C3a levels at follow-up in the LVD group is at least partly a consequence of the activation of the complement cascade by the underlying pathophysiological processes. These data, together with our findings of positive correlation between C3a and C3 in LVD but not cryptogenic stroke, indicate that in our study, brain

ischemia in the cryptogenic stroke patients was not caused by the acceleration of a less active and milder form of LVD, as proposed by Bang *et al.* [24,25]. Interestingly, plasma levels of hsCRP at three-month follow up were associated with LVD but not other ischemic stroke subtypes [20], again pointing to substantial differences in the underlying mechanisms between LVD and cryptogenic stroke.

CRP and C3 are acute phase proteins produced in large amounts in the liver in response to injury. Our correlation data indicate that plasma levels of these two proteins may be altered differently in response to ischemic stroke. Plasma C3 concentration reflects the net effect of C3 synthesis (regulated by pro-inflammatory cytokines) and its consumption due to the activation of the complement cascade. Complement activation in this setting can be triggered by CRP and most likely other activators that are released following ischemic brain injury. Thus measurements of hsCRP, C3 and C3a after stroke provide a complementary information about the patient's inflammatory status that might differ between strokes of different etiology.

Elevated plasma levels of C3 exhibited association with unfavourable outcome at three months follow-up as well as at two years after stroke. Follow-up but not acute levels of C3 were linked to unfavourable functional outcome but only in the LVD group. This difference in the predictive value of plasma C3 levels in the two stroke subtypes may be due to the difference in the underlying conditions. In the LVD group, atherosclerosis leads to chronic complement activation and is a strong determinant of outcome. In the cryptogenic group, the cause of stroke is unknown and the extent of systemic inflammatory response to cerebral ischemia may play a lesser role in outcome determination. In the cryptogenic group on the other hand, acute phase plasma C3a levels were

associated with greater disability at three months follow-up only in the univariate analysis and this association was lost after adjustment for age and sex. These findings are in line with data reported by Szeplaki and co-workers [16]. If infarct volume per se has an influence on C3 levels, this may contribute to our findings of an association to outcome. However, somewhat unexpectedly and in contrast to hsCRP, we did not observe any correlation between SSS and plasma C3 or C3a levels in the acute phase. Although SSS is only a crude measure of stroke severity that depends not only on the infarct volume but also on its location, this finding speaks against any major effect of infarct size on these parameters.

Results from a number of animal studies point to a dual role of inflammation and complement activation in brain ischemia. Genetic deficiency of C3 was associated with larger cerebral infarct after experimental permanent focal brain ischemia [7]. On the other hand, inhibition of complement activation or C3 deficiency reduced infarct volume and neurological impairments after transient cerebral ischemia in rodents [10-13, 26]. Blocking of C3a binding to its receptor reduced infarct volume assessed 24 hours after transient but not permanent cerebral ischemia in rodents [27]. Together with our findings, these results suggest that the role of complement proteins and their activation products in ischemic stroke may be rather complex and may depend on factors such as degree of reperfusion, stroke etiology and evaluation time point.

The present study has some limitations. As the SAHLIS project was not initially designed to study inflammatory responses, a detailed history of recent infections or chronic inflammatory events was not obtained. Moreover, the design of our study does not allow for a comparison of the absolute magnitude of complement response between LVD and cryptogenic

stroke patients. As we did not have access to direct measurements of infarct volume in the stroke patients, we cannot exclude that infarct volume confounded our results. Important to note is that the cryptogenic group in our study is not representative for all patients with cryptogenic stroke as patients with unfavourable outcome are overrepresented in the present sample compared to the whole cryptogenic group in SAHLSIS. Due to low numbers of participants, adjustment only for age and sex was possible in the analysis of outcome. Therefore, larger studies controlling for the effect of infections and enabling adjustment for the infarct volume, and additional risk factors are necessary.

In conclusion, our data suggest that plasma C3 and C3a levels are elevated in cryptogenic and LVD stroke. As high plasma C3 levels at three months after stroke were associated with unfavourable outcome in LVD but not cryptogenic stroke patients, the predictive value of complement proteins and their derivatives may depend on the ischemic stroke subtype.

Acknowledgements

The authors acknowledge the excellent technical assistance of Mrs. Ingrid Eriksson. This study was supported by grants by the Swedish Research Council (grant no 20116 to M. Pekna and 14605 to C. Jern), ALF Göteborg (grant 11267 to M. Pekna and 148861 to C. Jern), the Swedish Stroke Foundation, the Swedish Heart and Lung Foundation (20100256), the Göteborg Foundation for Neurological Research, and the Y. Land's, J. and B. Wennerström's Foundations for Neurological Research and as well as the R. and T. Söderberg's, T. Nilsson's, Emelle's, E. Jacobson's, W. and M. Lundgren's, R. and U. Amlöv's Foundations.

Disclosures

None.

REFERENCES

- 1 Lindsberg PJ, Grau AJ: Inflammation and infections as risk factors for ischemic stroke. *Stroke* 2003;34:2518-2532.
- 2 Danton GH, Dietrich WD: Inflammatory mechanisms after ischemia and stroke. *J Neuropathol Exp Neurol* 2003;62:127-136.
- 3 Stoll G, Bendszus M: Inflammation and atherosclerosis: Novel insights into plaque formation and destabilization. *Stroke* 2006;37:1923-1932.
- 4 Wahlgren CM, Zheng W, Shaalan W, Tang J, Bassiouny HS: Human carotid plaque calcification and vulnerability. Relationship between degree of plaque calcification, fibrous cap inflammatory gene expression and symptomatology. *Cerebrovasc Dis* 2009;27:193-200.
- 5 Arenillas JF, Alvarez-Sabin J, Molina CA, Chacon P, Fernandez-Cadenas I, Ribo M, Delgado P, Rubiera M, Penalba A, Rovira A, Montaner J: Progression of symptomatic intracranial large artery atherosclerosis is associated with a proinflammatory state and impaired fibrinolysis. *Stroke* 2008;39:1456-1463.
- 6 Worthmann H, Tryc AB, Goldbecker A, Ma YT, Tountopoulou A, Hahn A, Dengler R, Lichtinghagen R, Weissenborn K: The temporal profile of inflammatory markers and mediators in blood after acute ischemic stroke differs depending on stroke outcome. *Cerebrovasc Dis* 2010;30:85-92.
- 7 Rahpeymai Y, Hietala MA, Wilhelmsson U, Fotheringham A, Davies I, Nilsson AK, Zwirner J, Wetsel RA, Gerard C, Pekny M, Pekna M: Complement: A novel factor in basal and ischemia-induced neurogenesis. *EMBO J* 2006;25:1364-1374.
- 8 van Beek J, Nicole O, Ali C, Ischenko A, MacKenzie ET, Buisson A, Fontaine M: Complement anaphylatoxin c3a is selectively protective against nmda-induced neuronal cell death. *Neuroreport* 2001;12:289-293.
- 9 Mukherjee P, Pasinetti GM: Complement anaphylatoxin c5a neuroprotects through mitogen-activated protein kinase-dependent inhibition of caspase 3. *J Neurochem* 2001;77:43-49.
- 10 De Simoni MG, Storini C, Barba M, Catapano L, Arabia AM, Rossi E, Bergamaschini L: Neuroprotection by complement (c1) inhibitor in mouse transient brain ischemia. *J Cereb Blood Flow Metab* 2003;23:232-239.
- 11 Mocco J, Mack WJ, Ducruet AF, Sosunov SA, Sughrue ME, Hassid BG, Nair MN, Laufer I, Komotar RJ, Claire M, Holland H, Pinsky DJ, Connolly ES, Jr.: Complement component c3 mediates inflammatory injury following focal cerebral ischemia. *Circ Res* 2006;99:209-217.
- 12 Huang J, Kim LJ, Mealey R, Marsh HC, Jr., Zhang Y, Tenner AJ, Connolly ES, Jr., Pinsky

- DJ: Neuronal protection in stroke by an slex-glycosylated complement inhibitory protein. *Science* 1999;285:595-599.
- 13 Costa C, Zhao L, Shen Y, Su X, Hao L, Colgan SP, Stahl GL, Zhou T, Wang Y: Role of complement component c5 in cerebral ischemia/reperfusion injury. *Brain Res* 2006;1100:142-151.
- 14 Pedersen ED, Loberg EM, Vege E, Daha MR, Maehlen J, Mollnes TE: In situ deposition of complement in human acute brain ischaemia. *Scand J Immunol* 2009;69:555-562.
- 15 Lindsberg PJ, Ohman J, Lehto T, Karjalainen-Lindsberg ML, Paetau A, Wuorimaa T, Carpen O, Kaste M, Meri S: Complement activation in the central nervous system following blood-brain barrier damage in man. *Ann Neurol* 1996;40:587-596.
- 16 Szeplaki G, Szegedi R, Hirschberg K, Gombos T, Varga L, Karadi I, Entz L, Szeplaki Z, Garred P, Prohaszka Z, Fust G: Strong complement activation after acute ischemic stroke is associated with unfavorable outcomes. *Atherosclerosis* 2009;204:315-320.
- 17 Tamam Y, Iltumur K, Apak I: Assessment of acute phase proteins in acute ischemic stroke. *Tohoku J Exp Med* 2005;206:91-98.
- 18 Mocco J, Wilson DA, Komotar RJ, Sughrue ME, Coates K, Sacco RL, Elkind MS, Connolly ES, Jr.: Alterations in plasma complement levels after human ischemic stroke. *Neurosurgery* 2006;59:28-33; discussion 28-33.
- 19 Jood K, Ladenvall C, Rosengren A, Blomstrand C, Jern C: Family history in ischemic stroke before 70 years of age: The sahlgrenska academy study on ischemic stroke. *Stroke* 2005;36:1383-1387.
- 20 Ladenvall C, Jood K, Blomstrand C, Nilsson S, Jern C, Ladenvall P: Serum c-reactive protein concentration and genotype in relation to ischemic stroke subtype. *Stroke* 2006;37:2018-2023.
- 21 von Elm E, Altman DG, Egger M, Pocock SJ, Gotsche PC, Vandenbroucke JP: The strengthening the reporting of observational studies in epidemiology (strobe) statement: Guidelines for reporting observational studies. *J Clin Epidemiol* 2008;61:344-349.
- 22 Pedersen ED, Waje-Andreassen U, Vedeler CA, Aamodt G, Mollnes TE: Systemic complement activation following human acute ischaemic stroke. *Clin Exp Immunol* 2004;137:117-122.
- 23 Szeplaki G, Varga L, Fust G, Prohaszka Z: Role of complement in the pathomechanism of atherosclerotic vascular diseases. *Mol Immunol* 2009;46:2784-2793.
- 24 Bang OY, Lee PH, Joo SY, Lee JS, Joo IS, Huh K: Frequency and mechanisms of stroke recurrence after cryptogenic stroke. *Ann Neurol* 2003;54:227-234.
- 25 Bang OY, Lee PH, Yeo SH, Kim JW, Joo IS, Huh K: Non-cardioembolic mechanisms in cryptogenic stroke: Clinical and diffusion-weighted imaging features. *J Clin Neurol* 2005;1:50-58.
- 26 Atkinson C, Zhu H, Qiao F, Varela JC, Yu J, Song H, Kindy MS, Tomlinson S: Complement-dependent p-selectin expression and injury following ischemic stroke. *J Immunol* 2006;177:7266-7274.
- 27 Ducruet AF, Hassid BG, Mack WJ, Sosunov SA, Otten ML, Fusco DJ, Hickman ZL, Kim GH, Komotar RJ, Mocco J, Connolly ES: C3a receptor modulation of granulocyte infiltration after murine focal cerebral ischemia is reperfusion dependent. *J Cereb Blood Flow Metab* 2008;28:1048-1058.

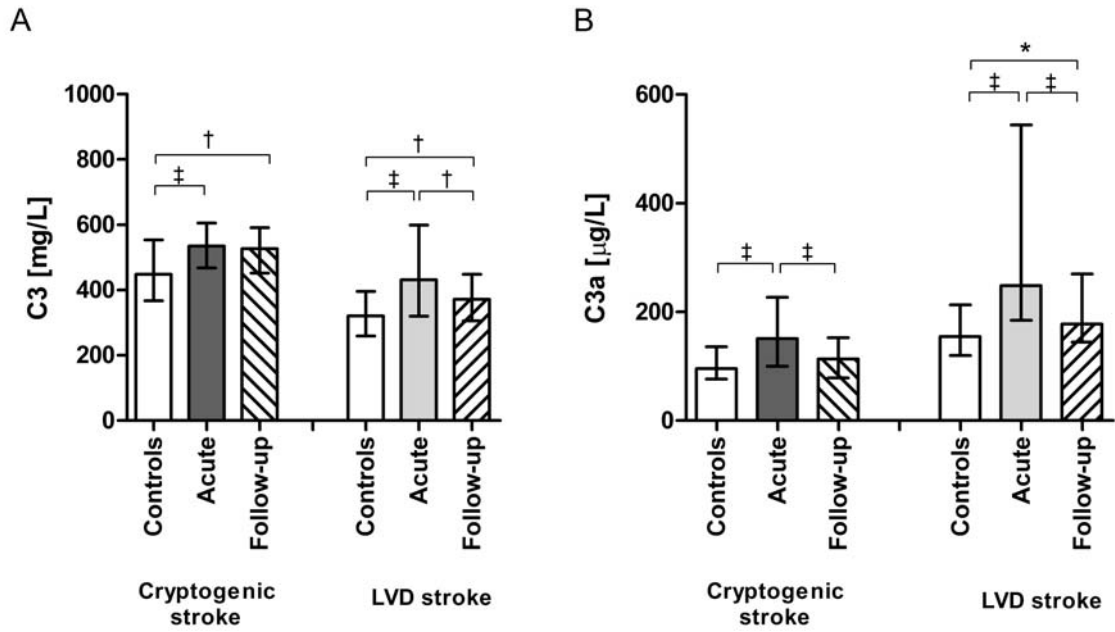


Figure 1. Median levels of plasma C3 (A) and C3a (B) with error bars representing interquartile ranges. Differences between control versus acute or follow-up measurements were examined by Mann-Whitney *U* test and acute versus follow-up by Wilcoxon signed rank test. * $P < 0.05$; † $P < 0.01$; ‡ $P < 0.001$.

Table 1. Demographic and clinical characteristics of the study population

	Cryptogenic stroke		LVD stroke		
	Controls (n=40)	Patients (n=79)	Controls (n=39)	Patients (n=73)	
Median age, years (IQ-R)	59 (53-62)	57 (51-63)	60 (56-63)	60 (57-64)	
Male sex, n (%)	25 (62)	49 (62)	30 (77)	54 (74)	
Hypertension, n (%)	16 (40)	49 (62)*	14 (36)	44 (60)*	
Diabetes mellitus, n (%)	5 (12)	13 (16)	5 (13)	25 (34)*	
Current smoking, n (%)	5 (12)	31 (39)†	9 (23)	39 (53)†	
Hyperlipidemia, n (%)	21 (52)	53 (69)	29 (74)	53 (82)	
BMI, median (IQ-R)	25.38 (22.29-28.50)	27.71 (24.03-28.23)	26.52 (25.33-28.91)	26.12 (24.05-28.91)	
SSS acute, median (IQ-R)	...	52 (31-55)	...	52 (33-57)	
hsCRP [mg/L], median (IQ-R)					
acute	1.98 (0.89-4.13)	2.76 (1.11-7.91)	1.49 (0.96-2.37)	4.66 (1.86-13.28)‡	Differences between cases and
follow-up	...	1.81 (1.03-4.24)	...	3.64 (1.58-9.28)‡	

controls were examined with χ^2 test for proportions or Mann-Whitney U test for continuous variables.

* $P < 0.05$; † $P < 0.01$; ‡ $P < 0.001$.

LVD - large vessel disease stroke; IQ-R – inter-quartile range; BMI – body mass index; SSS – Scandinavian Stroke Scale score; hsCRP – high sensitivity C-reactive protein.

Table 2. Correlations between plasma levels of C3 and C3a, hsCRP, and SSS

	C3a follow-up	C3 acute	hsCRP [§]	SSS acute
Cryptogenic stroke				
C3 acute	---	---	0.446 ‡	- 0.203
C3 follow-up	- 0.078	0.670 ‡	0.364 †	- 0.138
C3a acute	0.502 †	- 0.236 *	0.167	- 0.043
C3a follow-up	---	---	0.278 *	- 0.070
LVD stroke				
C3 acute	---	---	0.136	- 0.024
C3 follow-up	0.141	0.438 ‡	0.141	- 0.307 *
C3a acute	0.526 ‡	0.527 ‡	0.376 †	- 0.070
C3a follow-up	---	---	0.251 *	0.101
Controls				
C3	---	---	0.316 †	---
C3a	---	- 0.220	0.043	---

Values given represent Spearman's correlation coefficients (ρ) with significance levels denoted as follows: * $P < 0.05$; † $P < 0.01$; ‡ $P < 0.001$.

§ - variable measured at the same time point as the respective indicated measurements of C3 or C3a.

--- indicates non-meaningful correlation; hsCRP – high sensitivity C-reactive protein; SSS – Scandinavian Stroke Scale score; LVD – large vessel disease.

Table 3. Univariate and multivariate ORs with 95 % CIs of association between C3 and C3a levels and stroke

Tertiles of C3 and C3a	Controls, n	Cases, n	Univariate OR (95% CI)	Multivariate Model 1 OR (95% CI)
Cryptogenic stroke				
C3 acute (all)	(40)	(75)		
Lower third	22	18	1.0 (reference)	1.0 (reference)
Middle third	9	26	3.53 (1.32-9.42)*	3.95(1.32-11.80)*
Upper third	9	31	4.21 (1.60-11.09)†	4.45 (1.51-12.87)†
C3 follow-up (all)	(40)	(77)		
Lower third	21	19	1.0 (reference)	1.0 (reference)
Middle third	9	28	3.44 (1.30-9.11)*	4.34 (1.48-12.75)†
Upper third	10	30	3.32 (1.29-8.55)*	2.70 (0.94-7.71)
C3a acute (all)	(40)	(75)		
Lower third	21	19	1.0 (reference)	1.0 (reference)
Middle third	13	26	2.21 (0.89-5.49)	2.47 (0.87-7.09)
Upper third	6	30	5.53 (1.89-16.48)†	8.99 (2.53-31.90)‡
C3a follow-up (all)	(40)	(77)		
Lower third	16	24	1.0 (reference)	1.0 (reference)
Middle third	12	26	1.44 (0.57-3.67)	1.39 (0.43-3.89)
Upper third	12	27	1.50 (0.59-3.80)	1.18 (0.42-3.27)
LVD stroke				
C3 acute (all)	(39)	(71)		
Lower third	19	19	1.0 (reference)	1.0 (reference)
Middle third	14	23	1.64 (0.66-4.12)	1.91 (0.64-5.71)
Upper third	6	29	4.83 (1.63-14.30)†	5.93 (1.67-20.87)†
C3 follow-up (all)	(39)	(65)		
Lower third	19	19	1.0 (reference)	1.0 (reference)
Middle third	10	19	1.80 (0.66-4.89)	1.96 (0.60-6.42)
Upper third	10	27	2.70 (1.03-7.09)*	3.26 (1.04-10.23)*
C3a acute (all)	(39)	(71)		
Lower third	23	15	1.0 (reference)	1.0 (reference)
Middle third	12	25	3.19 (1.24-8.23)*	2.46 (0.84-7.19)
Upper third	4	31	11.88 (3.48-40.56)‡	11.55 (2.98-44.79)‡
C3a follow-up (all)	(39)	(64)		
Lower third	19	19	1.0 (reference)	1.0 (reference)
Middle third	12	22	1.83 (0.71-4.73)	2.05 (0.69-7.16)
Upper third	8	23	2.87 (1.03-8.02)*	3.72 (1.16-11.94)*

OR – odds ratio; CI – confidence intervals; LVD – large vessel disease.

Model 1: adjusted for age, sex, hypertension, smoking and diabetes; * $P < 0.05$; † $P < 0.01$;

‡ $P < 0.001$.

Table 4. Cut-offs for tertiles of plasma C3 and C3a levels

	Cryptogenic stroke		LVD stroke	
	acute	follow-up	acute	follow-up
C3 (mg/L)				
Lower third	<467.88	<451.70	<320.46	<316.78
Middle third	467.88 - 566.67	451.70 - 557.21	320.46 - 450.27	316.78 - 391.52
Upper third	>566.67	>557.21	>450.27	>391.518
C3a (µg/L)				
Lower third	<100.58	< 87.69	<171.78	<152.07
Middle third	100.58 - 175.22	87.69 - 131.11	171.78 - 260.28	152.07 - 220.27
Upper third	>175.22	>131.11	>260.28	>220.27

LVD – large vessel disease.

Table 5. Selected[§] univariate and multivariate ORs with 95 % CIs of association between C3 and C3a levels and functional outcome

	Tertiles of C3 and C3a	Favourable outcome, n	Unfavourable outcome, n	Univariate OR (95% CI)	Multivariate Model 2 OR (95% CI)
Outcome at three months	Cryptogenic stroke				
	C3a acute (all)	(47)	(28)		
	Lower third	15	4	1.0 (reference)	1.0 (reference)
	Middle third	17	9	1.99 (0.51-7.79)	1.97 (0.50-7.76)
	Upper third	15	15	3.75 (1.01-13.96)*	3.74 (0.99-14.04)
	LVD stroke				
	C3 follow-up (all)	(47)	(17)		
	Lower third	17	2	1.0 (reference)	1.0 (reference)
Middle third	14	5	3.04 (0.51-18.10)	3.25 (0.53-20.04)	
Upper third	16	10	5.31 (1.01-28.07)*	5.56 (1.03-29.93)*	
Outcome at two years	Cryptogenic stroke				
	C3a acute (all)	(52)	(22)		
	Lower third	16	3	1.0 (reference)	1.0 (reference)
	Middle third	18	8	2.37(0.53-10.50)	2.35 (0.51-10.71)
	Upper third	18	11	3.25(0.76-13.80)	3.15 (0.73-13.69)
	LVD stroke				
	C3 follow-up (all)	(44)	(21)		
	Lower third	16	3	1.0 (reference)	1.0 (reference)
Middle third	14	5	1.90 (0.38-9.44)	1.75 (0.35-8.82)	
Upper third	14	13	4.95 (1.17-21.03)*	4.75 (1.11-20.30)*	

[§] - remaining non-significant results are not shown; * $P < 0.05$; OR - odds ratio; CI - confidence intervals; LVD - large vessel disease. Model 2: adjusted for age and sex.