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Proinflammatory protein signatures in cryptogenic and large artery atherosclerosis stroke

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Objectives: The cause of ischemic stroke remains unknown, cryptogenic, in 25% of young and middle-aged patients. We hypothesized that if atherosclerosis is prominent in cryptogenic stroke, it would have a similar proinflammatory protein signature as large artery atherosclerosis (LAA) stroke.

Materials & Methods: Blood was collected in the acute phase and after 3 months from cryptogenic (n = 162) and LAA (n = 73) stroke patients aged 18–69 years and once from age-matched controls (n = 235). Cryptogenic stroke was divided into Framingham Risk Score (FRS) quartiles to compare low and high risk of atherosclerosis. Plasma concentrations of 25 proteins were analyzed using a Luminex multiplex assay. The discriminating properties were assessed with discriminant analysis and C-statistics.

Results: We identified proteins that separated cryptogenic and LAA stroke from controls (area under the curves, AUCs ≥ 0.85). For both subtypes, RANTES, IL-4, and IFN-γ contributed the most at both time points. These associations were independent of risk factors of atherosclerosis. We also identified proteins that separated cryptogenic strokes in the lowest quartile of FRS from those in the highest, and from LAA stroke (AUCs ≥ 0.76), and here eotaxin and MCP-1 contributed the most.

Conclusions: The protein signature separating cases from controls was different from the signature separating cryptogenic stroke with low risk of atherosclerosis from those with high risk and from LAA stroke. This suggests that increased RANTES, IL-4, and IFN-γ in stroke may not be primarily related to atherosclerosis, whereas increased eotaxin and MCP-1 in cryptogenic stroke may be markers of occult atherosclerosis as the underlying cause.

KEYWORDS
atherosclerosis, case-control study, cryptogenic stroke, cytokines, etiology, inflammation, ischemic stroke

1 | INTRODUCTION

In young and middle-aged ischemic stroke patients, the underlying etiology cannot be identified in as many as 25% of the cases, despite extensive clinical work-up.[1–3] In this group, comprising the so-called cryptogenic strokes, atherosclerosis currently thought to be unrelated to the stroke[4] has been suggested to be one possible etiology, although the group is likely to be heterogenous.[4] Inflammation has been implicated in all major etiologic subtypes of ischemic stroke.[5] The association seems to be strongest in large artery atherosclerosis stroke (LAA), which is plausible since atherosclerosis is believed to be driven by inflammation.[6] However, after taking risk factors for atherosclerosis into account, the associations with proinflammatory biomarkers such as C-reactive protein are weakened in all major subtypes.[5] This can indicate that atherosclerosis is important not only in LAA stroke but also in several other subtypes.

We hypothesized that if atherosclerosis is a prominent feature of cryptogenic stroke, then patients with cryptogenic stroke would display a similar proinflammatory plasma protein signature as patients with LAA stroke. To investigate this hypothesis, we examined profiles of proteins associated with inflammation in cryptogenic stroke, in LAA stroke, and in healthy controls. We also stratified the cryptogenic group according to the number of risk factors for atherosclerosis in order to investigate whether cryptogenic stroke cases with multiple risk factors of atherosclerosis have a protein signature more resembling that of LAA stroke than that of cryptogenic stroke with few risk factors of atherosclerosis.

2 | MATERIALS AND METHODS

2.1 | Study population

The study sample comprised participants from the Sahlgrenska Academy Study on Ischemic Stroke (SAHLSIS), the design of which has been reported previously.[1] Briefly, patients aged 18–69 years (n = 600) who presented with ischemic stroke were recruited consecutively at four stroke units in western Sweden. Patients were examined in the acute phase and at a follow-up visit after three months. They were classified into etiologic subtypes by TOAST criteria with minor modifications as described in the Supporting Information available online. In the present study, all patients classified as cryptogenic (n = 162) or large artery atherosclerosis (LAA) stroke (n = 73) were included along with their matched controls (n = 235). Cryptogenic stroke was defined as cases in whom no cause was identified despite full clinical investigation. Maximum stroke severity within the first 7 days after the stroke was scored using the Scandinavian Stroke Scale (SSS). Like the National Institutes of Health Stroke Scale (NIHSS), SSS quantifies the impairment caused by a stroke, but differs in the direction of measurement. Additional details and definitions of risk factors of atherosclerosis can be found in the Supporting Information available online.

As described in the Supporting Information, all cases in the present study underwent Doppler ultrasonography, transcranial Doppler ultrasonography, and/or magnetic resonance or computed tomography angiography of the brain arteries. As these investigations were performed within clinical routine, we only have information on the presence and absence of stenosis >50% (North American Symptomatic Carotid Endarterectomy Trial, NASCET). In order to define one group with low risk and one with high risk of atherosclerosis within the cryptogenic group, we used the Framingham Risk Score (FRS).[7] This score was originally developed to predict the 10-year risk of coronary heart disease (CHD) in patients without overt CHD, but has later been shown to be associated with atherosclerotic burden in middle-aged adults,[8] and to correlate well with direct measures of carotid artery atherosclerosis.[9] Cryptogenic stroke cases were divided into four quartiles of FRS. Cases with FRS in the lower quartile (Q1) were considered as having a low risk, and those in the upper quartile (Q4) a high risk of atherosclerosis.

Controls were selected from the community as previously described.[1] They were examined once, and excluded if they reported a history of stroke, CHD, peripheral artery disease, rheumatic, other autoimmune or proinflammatory disorder, or if they had signs of ischemic heart disease on resting ECG. Included controls were individually matched to cases for age (±1 year), sex, and geographic area of residence.

Informed consent was obtained from all participants prior to enrollment. For participants who were unable to communicate, consent was obtained from their next-of-kin. The study was approved by the Ethics Committee of the University of Gothenburg and conforms with the World Medical Association Declaration of Helsinki.

2.2 | Blood sampling and plasma protein measurements

For patients, blood was collected in the acute phase within 10 days of the index stroke (median 5 days, IQR 3–6 days), and at the three-month follow-up (median 100 days, IQR 94–110 days). For the controls, blood sampling was performed once. On all occasions, venous blood was collected between 08.30 and 10.30 AM after overnight fasting. In controls and in patients at three-month follow-up, blood was not collected if the participant had signs of infection. Blood was drawn in tubes containing 10% by volume

ethylenediaminetetraacetic acid (EDTA). Plasma was isolated within two hours by centrifugation at 2000 *g* at 4°C for 20 min, aliquoted, and stored at −80°C.

Plasma concentrations of 25 cytokines and chemokines were measured with the Human Antibody Bead 25-plex kit for the Luminex platform (Life Technologies Corporation, Carlsbad, California, USA) according to the manufacturer's instructions. The analytes are listed in the Supporting Information. All analyses were performed by a board-certified laboratory technician who was blinded to the clinical information. Samples from the same case participant and their matched control were grouped on the same plate. To accommodate for inter-plate variation, the individual protein levels were divided by the protein median of the current plate and multiplied by the protein median of all plates. Values below the range of the standard curve were accepted if they were above 75% of the lowest concentration in the standard curve; otherwise, they were assigned a value corresponding to 75% of the lowest concentration in the standard curve. Missing values per analyte was ≤3.0% for controls, ≤5.5% for patients in the acute phase and ≤12.8% for patients at follow-up. Additional details can be found in the Supporting Information available online.

2.3 | Statistical analyses

Differences in clinical characteristics between paired cases and controls for continuous variables and for proportions were assessed with Wilcoxon's signed rank tests and McNemar's tests, respectively. Differences in proportions of plasma protein concentrations above the lowest concentration in the standard curve between paired cases and controls were assessed with McNemar's tests. For analytes with >75% of samples within the range of the standard curve, medians and confidence intervals were calculated and tests of differences were performed with Wilcoxon's signed rank test.

To compare plasma protein signatures between different groups, orthogonal projections to latent structures-discriminant analysis (OPLS-DA)[10] was used. The data distribution for several proteins was highly positively skewed. Data were therefore logarithmically transformed and standardized, before OPLS-DA. Only analytes with more than 75% of samples having values within the range of the standard curve were included. The discriminating power of the OPLS-DA models and of individual proteins was evaluated by plotting receiver operating characteristics (ROC) curves and calculating the areas under the curves (AUC). Bootstrap methods were used for significance testing and for construction of confidence intervals. For the discriminating abilities, AUCs in the ranges 0.9–1 were considered as excellent, 0.8–0.9 as good, 0.7–0.8 as fair, 0.6–0.7 as poor, and 0.5–0.6 as failed. To identify the strongest class-discriminating proteins, variable importance on projection (VIP) values from the OPLS-DA models was used.[11] For predictive models, cutoff values for VIP around 0.7–0.8 have been shown to work well for discrimination between important and unimportant predictors.[12]

The proteins that contributed the most to the discrimination between patients and controls in the OPLS-DA were subject to additional analyses with single variable conditional logistic regression, and multivariable ordinary logistic regression. The multivariable models included age, sex, smoking, hypertension, diabetes mellitus, low-density lipoprotein, and waist-hip-ratio as covariates. To further investigate the influence of atherosclerosis, the protein signatures of cryptogenic stroke patients with low versus high risk of atherosclerosis as defined above (i.e., Q1 vs Q4 FRS) were compared with each other and with LAA stroke patients using OPLS-DA.

OPLS-DA was performed in the statistical software package SIMCA, version 13.0 (Umetrics AB, Umeå, Sweden). All other statistics were performed in R (version 3.0.0), the R Foundation for Statistical Computing, with the package pROC (version 1.7.1). Two-tailed tests were used throughout.

TABLE 1 Baseline characteristics for the study groups

	LAA stroke		Cryptogenic stroke	
	Cases (*n* = 73)	Controls (*n* = 73)	Cases (*n* = 162)	Controls (*n* = 162)
Age, mean (SD)	59 (8)	59 (8)	53 (12)	53 (12)
Male sex, no. (%)	54 (74)	54 (74)	95 (59)	95 (59)
Current smoking, no. (%)	39 (53)***	16 (22)	60 (37)**	36 (22)
Hypertension, no. (%)	44 (60)***	23 (32)	87 (54)***	52 (32)
Diabetes mellitus, no. (%)	25 (34)***	5 (7)	23 (14)*	10 (6)
Hyperlipidemia, no. (%)	53 (73)	57 (78)	107 (66)*	95 (59)
WHR ×100, mean (SD)	96 (6)	94 (8)	94 (7)***	91 (7)
SSS, median (IQR)	52 (33–57)	N/A	54 (45–57)	N/A

Notes: Asterisks indicate significant differences between paired cases and controls as calculated with Wilcoxon signed rank test for continuous variables, and with McNemar's test for proportions.

Abbreviations: LAA, large artery atherosclerosis; SSS, Scandinavian stroke scale (maximum score is 58, indicating full function); WHR, waist-hip ratio.

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

3 | RESULTS

Characteristics for the study groups are summarized in Table 1. Compared with controls, cases were more likely to smoke and have hypertension and diabetes mellitus. In addition, for cryptogenic stroke, cases were more likely to have hyperlipidemia and a higher waist-hip ratio than controls.

For most plasma proteins, both the proportion of samples above the lowest concentration in the standard curve and the median plasma concentration were higher in both cryptogenic and LAA stroke, than in matched controls, at both time points (Tables S1 and S2 in the Supporting Information). For 16 of the 25 proteins, the concentrations were above the lowest concentration in the standard curve in more than 75% of samples. These proteins were subject to further statistical analyses.

As a first step, we analyzed differences in protein signatures using OPLS-DA followed by ROC-analysis. For differences between cases and controls in the acute phase, the analyses showed good separation, in both LAA and in cryptogenic stroke, with AUCs of 0.87 (95% CI: 0.81–0.94) and 0.89 (95% CI: 0.85–0.92) respectively, Figure 1. Also, at follow-up, the separation between cases and controls, in both LAA and cryptogenic stroke was good with AUCs of 0.85 (95% CI: 0.78–0.92) and 0.87 (95% CI: 0.82–0.91) respectively, Figure 2. The protein signatures were similar for both subtypes and at both time points. VIP plots revealed that the proteins with the largest contributions to the separation were as follows: RANTES, IL-4, and IFN-γ, followed by MIP-1α, and IL-17. As can be seen in Figures 1 and 2, the OPLS-DA models were not superior to the strongest contributing proteins individually, in separating the groups in either of the subtypes, and at either of

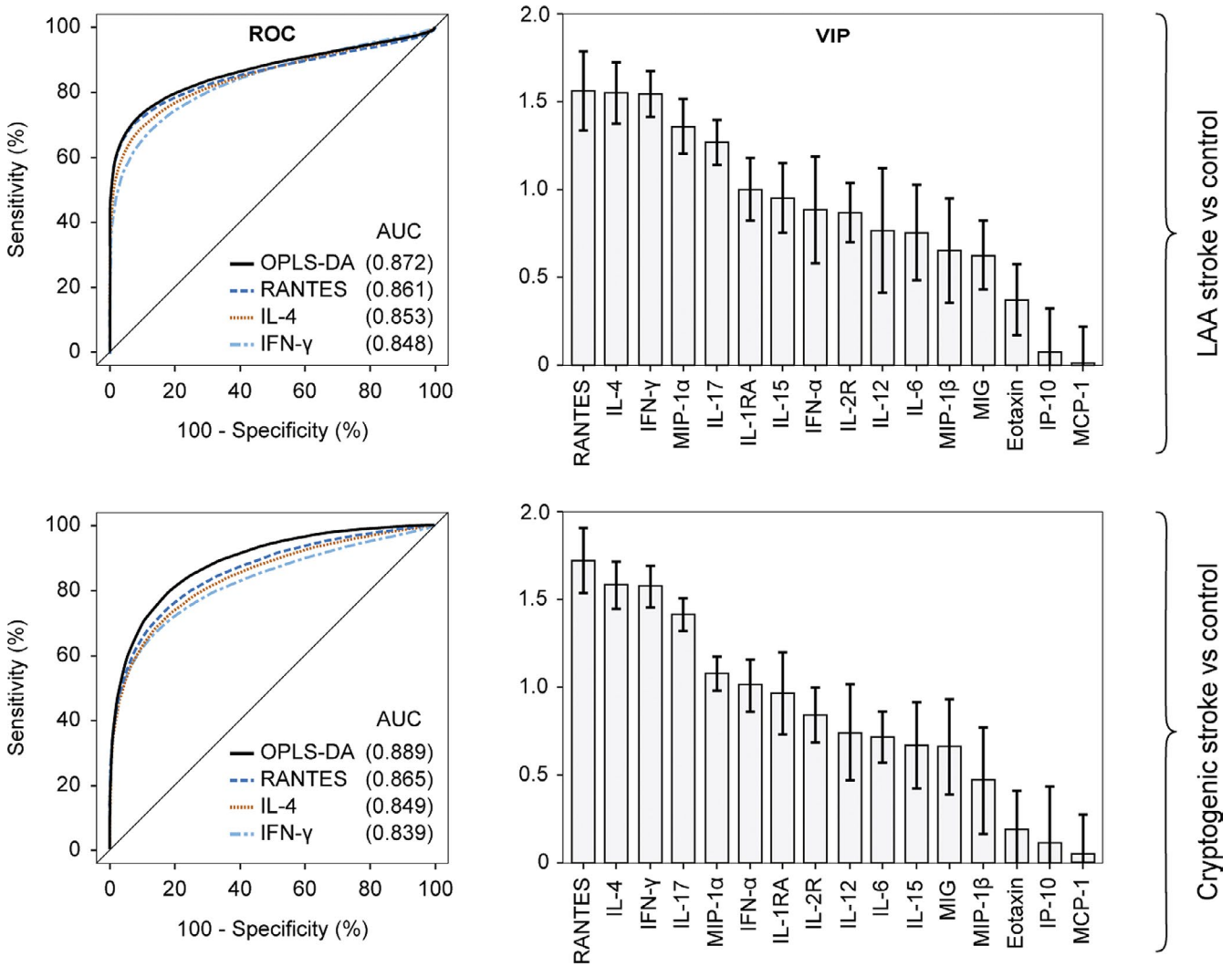

FIGURE 1 ROC curves showing the separation between patients in the acute phase and controls for LAA and cryptogenic stroke. The different ROC curves are for the OPLS-DA models and for the three individual plasma proteins contributing the most to the separation as indicated by the VIP plots. No significant differences were found between the AUCs of the OPLS-DA models and the individual proteins in the ROC diagrams. Error bars in the VIP diagrams represent 95% confidence intervals. AUC, area under the curve; LAA, large artery atherosclerosis; OPLS-DA, orthogonal projections to latent structures-discriminant analysis; ROC, receiver operating characteristic; VIP, Variable importance for the projection

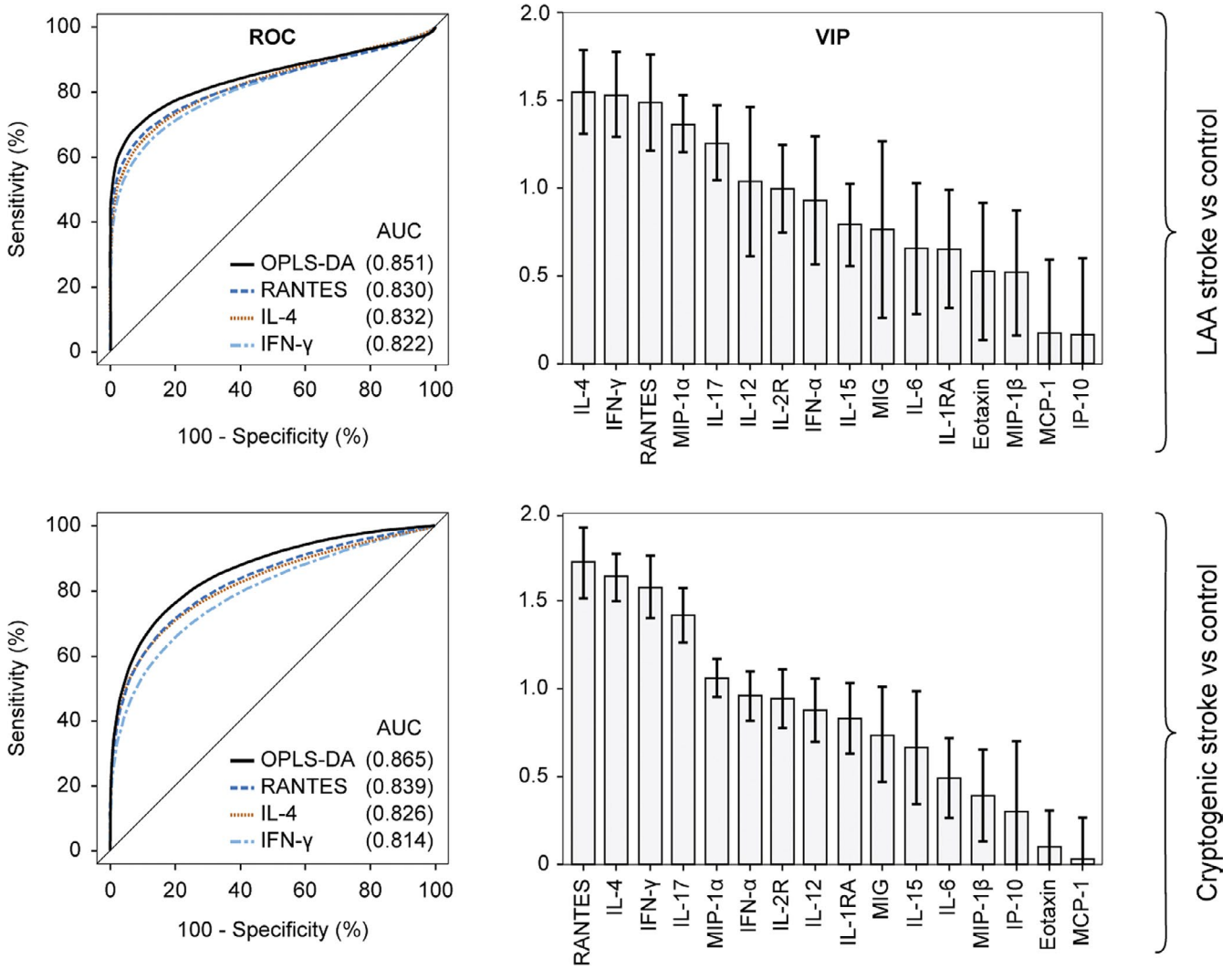

FIGURE 2 ROC curves showing the separation between patients at 3-month follow-up and controls for LAA and cryptogenic stroke. The different ROC curves are for the OPLS-DA models and for the three individual plasma proteins contributing the most to the separation as indicated by the VIP plots. No significant differences were found between the AUCs of the OPLS-DA models and the individual proteins in the ROC diagrams. Error bars in the VIP diagrams represent 95% confidence intervals. AUC, area under the curve; LAA, large artery atherosclerosis; OPLS-DA, orthogonal projections to latent structures-discriminant analysis; ROC, receiver operating characteristic; VIP, Variable importance for the projection

the time points. Analyses showed poor separation of acute phase and at follow-up samples, both in LAA (AUC 0.67, 95% CI: 0.57–0.76) and in cryptogenic stroke (AUC 0.60, 95% CI: 0.54–0.67), see Figure S1 in the Supporting Information. Also, for differences between protein signatures in cryptogenic and LAA stroke, analyses showed poor separation both in the acute phase and at follow-up, with AUCs of 0.66 (95% CI: 0.59–0.73) and 0.65 (95% CI: 0.57–0.73) respectively, see Figure S2 in the online Supporting Information.

We next compared concentrations of the individual proteins in the acute phase with those at follow-up. For all patients, significant differences were found for IFN-γ, IL-4, IL-6, IL-1RA, IP-10, MIP-1α, and RANTES. However, only IL-1RA (p = 0.005) remained significantly higher in the acute phase compared to follow-up after Bonferroni correction. We also investigated the influence of stroke severity assessed by the SSS, as a crude proxy of infarct size, on the individual protein concentrations. Here, the only significant association was with IL-6, which increased with level of impairment, and only before Bonferroni correction.

To account for possible confounders, the three proteins contributing the most to the separation between patients and controls (RANTES, IL-4, and IFN-γ) were analyzed with logistic regression. The odds ratios for ischemic stroke were 2.3–3.6 with each two-fold increase in concentration of the individual protein at both time points (Table 2). These associations were retained when including risk factors of atherosclerosis in the models (Table 2). The study was not designed to investigate the influence of antiplatelet drugs and statins on cytokine levels. However, to gain insight into this, patients on antiplatelet medication (N = 189) were compared with patients on anticoagulant medication (N = 25), and patients with statins (N = 91)

TABLE 2 Odds ratios and 95% confidence intervals for ischemic stroke per each two-fold increase in plasma protein concentration

	Rantes	IFN-γ	IL–4
Univariate			
Acute			
LAA	2.27 (1.59–3.23)***	2.69 (1.71–4.24)***	3.02 (1.79–5.09)***
Cryptogenic	2.96 (2.08–4.22)***	2.34 (1.76–3.09)***	3.08 (2.07–4.58)***
Follow-up			
LAA	2.67 (1.69–4.07)***	2.57 (1.62–4.07)***	3.57 (1.87–6.81)***
Cryptogenic	3.05 (2.11–4.41)***	2.27 (1.73–2.99)***	3.14 (2.11–4.66)***
Acute			
LAA	2.63 (1.87–3.98)***	2.60 (1.84–3.97)***	3.44 (2.18–6.12)***
Cryptogenic	3.61 (2.71–5.03)***	2.87 (2.24–3.83)***	3.59 (2.62–5.14)***
Follow-up			
LAA	2.37 (1.72–3.49)***	2.48 (1.77–3.76)***	3.01 (1.99–5.01)***
Cryptogenic	3.13 (2.39–4.27)***	2.38 (1.91–3.05)***	3.22 (2.39–4.54)***

Notes: The multivariable models included age, sex, smoking, hypertension, diabetes mellitus, low-density lipoprotein cholesterol, and waist-hip ratio at baseline as covariates. Asterisks indicate significant differences between cases and controls.

Abbreviation: LAA, large artery atherosclerosis.

***$p < 0.001$.

were compared with those without (N = 144). Comparing the antiplatelet and anticoagulation treatment groups, no significant differences were found. For the statin vs non-statin treatment analyses, the only significant difference was for IL-6 (p = 0.042) and only before Bonferroni correction.

As a final step, the cryptogenic stroke cases with low and high risk of atherosclerosis (ie, FRS Q1 vs Q4) were compared with each other and with LAA stroke. The median FRS for cryptogenic stroke at follow-up was 10% (IQR 4–17%), as compared with 22% (IQR 15–31%) in LAA. OPLS-DA followed by ROC-analyses at follow-up showed poor separation between cryptogenic stroke with FRS in the higher quartile and LAA stroke, with an AUC of 0.67 (95% CI: 0.55–0.79). In contrast, the separation between cryptogenic stroke with FRS in the lower quartile and LAA stroke was good with an AUC of 0.84 (95% CI: 0.76–0.93), the separation between cryptogenic stroke with FRS in the lower and higher quartile was fair with an AUC of 0.76 (95% CI: 0.67–0.84), and in both cases eotaxin and MCP-1 contributed the most to this separation (Figure 3).The median concentration (95% CI) in pg/ml for eotaxin in cryptogenic stroke with FRS in the lower and higher quartile, and in LAA stroke were 51.9 (44.6–59.0), 82.2 (67.7–97.1), and 82.8 (74.3–94.2) respectively. The corresponding values for MCP-1 were 239 (201–296), 315 (277–358), and 308 (284–338), respectively.

4 | DISCUSSION

In this explorative study of circulating proinflammatory protein signatures in cryptogenic and LAA stroke, we found that the signatures of the two etiologic subtypes resembled each other, both in the acute phase and after three months. In both subtypes, the plasma proteins provided good separation from controls, at both time points. The plasma proteins that contributed the most to this separation were RANTES, IL-4, and IFN-γ. We also found that in cryptogenic stroke with low risk of atherosclerosis defined by FRS, the proteins provided good separation from LAA stroke, and fair separation from cryptogenic stroke with high risk of atherosclerosis. Here, eotaxin and MCP-1 contributed the most to the separation. For cryptogenic stroke with high risk of atherosclerosis on the other hand, the separation from LAA stroke was poor.

The fact that the protein signatures for cryptogenic and LAA stroke at the two time points resembled each other to such a high degree was somewhat surprising. We had expected that the levels in the acute phase would be elevated compared to those after three months reflecting a response to the acute ischemic lesion superimposed on the pre-stroke levels. The question then arises, whether the elevation at 3 months post-stroke was a lasting effect of the ischemic lesion *per se*, or was present already before the ischemic stroke, or is a combination of both. There are limited data on time series of circulating cytokines and chemokines after ischemic stroke, especially beyond the first few weeks.[13] Some data point toward the elevation being an effect of the ischemic event. There are, for instance, reports on a substantial T-cell response after stroke.[14,15] Such a response for RANTES was found in one case-control study on ischemic stroke from the Fukuoka Stroke Registry, where levels of RANTES increased from day 0 to day 14 after the ischemic event.[16] Similarly, a small South Korean case-control study reported that IL-4 levels were elevated in the acute phase after ischemic stroke.[17] Other data point toward the elevation being present prior to the ischemic event. In the large prospective PRIME study, it was found that elevated concentrations of RANTES predicted incident ischemic stroke.[18] Further, although the Fukuoka study showed that

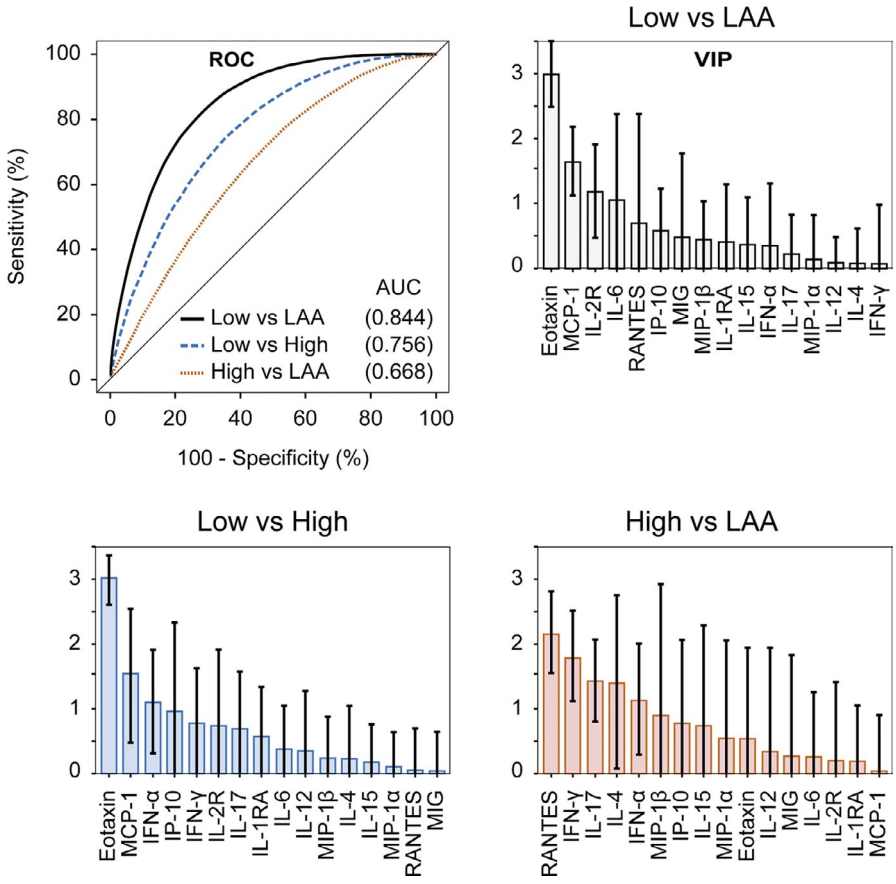

FIGURE 3 ROC curves showing the separation between cryptogenic stroke with high risk of atherosclerosis, cryptogenic stroke with low risk of atherosclerosis, and LAA stroke, at the three-month follow-up, achieved by OPLS-DA. The VIP plots show the contribution of the individual plasma proteins to the OPLS-DA models. Cryptogenic stroke with low risk of atherosclerosis was defined as a Framingham risk score at baseline below the lower quartile, whereas high risk was defined as a Framingham risk score above the upper quartile. Error bars in the VIP diagrams represent 95% confidence intervals. AUC, area under the curve; LAA, large artery atherosclerosis; OPLS-DA, orthogonal projections to latent structures-discriminant analysis; ROC, receiver operating characteristic; VIP, Variable importance for the projection

RANTES was increased in the acute phase, the concentration had declined substantially by day 90, but remained elevated in patients compared with controls.[16] Also, the South Korean study mentioned above reported that IFN-γ was not elevated in the acute phase of ischemic stroke,[17] whereas some studies of cardiovascular disease have found increased levels of IFN-γ.[19,20] We found no significant associations between RANTES, IFN-γ, and IL-4 levels and stroke severity in our study. The only plasma protein that was significantly associated with stroke severity was IL-6, but only before Bonferroni correction. However, there are studies indicating that IL-6 is in fact associated with infarct size.[21–23] In line with this, previous studies have found an association between CRP, which is induced by IL-6,[24] and infarct size.[5,21] From our results, and in light of the aforementioned studies, we suggest that the elevated levels of RANTES, IFN-γ, and IL-4 at 3 months post-stroke mainly reflect pre-stroke levels, although we recognize that they may also be affected by the ischemic lesion *per se*.

We next compared the cryptogenic stroke group with FRS in the lower quartile with the cryptogenic stroke group with FRS in the higher quartile (i.e., cryptogenic stroke with low versus high risk of atherosclerosis) and found a protein signature separating these groups. This signature also differentiated cryptogenic stroke group with FRS in the lower quartile from LAA stroke. Here, eotaxin (also known as eosinophil chemotactic protein, encoded by the CCL11 gene) and MCP-1 (also known as chemokine C-C motif ligand 2, CCL2) contributed the most to the separations. Both eotaxin and MCP-1 were elevated in LAA stroke and cryptogenic stroke with FRS in the higher quartile compared to cryptogenic stroke with FRS in the lower quartile, and they have previously been implicated in atherosclerosis. Eotaxin is overexpressed in carotid artery atherosclerotic plaques,[25] has been suggested to associate with carotid intima-media thickness,[26] and a variant within the CCL11 gene has been reported to associate with the development of restenosis after percutaneous coronary interventions.[27] In the PRIME study, systemic levels of eotaxin were independently associated with ischemic stroke, but not with CHD.[18] Not only eotaxin, but also MCP-1 expression is upregulated in atherosclerotic plaques.[28,29] MCP-1 has been found in atherosclerotic coronary arteries[30] and has been suggested to have a non-cholesterol-mediated effect on atherogenesis.[31] Interestingly, a recent Mendelian randomization study showed

an association between a genetic predisposition to elevated circulating levels of MCP-1 and increased risk of ischemic stroke, in particular with LAA stroke and cardioembolic stroke, and with CHD and myocardial infarction.[32] This study also performed a meta-analysis showing higher MCP-1 levels in patients with stroke compared with controls.[32] Thus, our results in conjunction with previous findings support the suggestion that atherosclerosis currently thought to be unrelated to cryptogenic stroke may in fact be the underlying etiology in a subgroup of cryptogenic stroke patients. This assumption is supported by previous findings of a high prevalence of traditional cardiovascular risk factors in young onset ischemic stroke.[2]

The proteins that separated cryptogenic stroke patients with low risk of atherosclerosis from those with high risk of atherosclerosis and from LAA stroke were different from those separating stroke patients of both subtypes from controls, in which RANTES, IL-4, and IFN-γ had the largest influence. Further, results from our multivariable logistic regression analyses showed that the associations between ischemic stroke and RANTES, IL-4, and IFN-γ in both subtypes were independent of risk factors of atherosclerosis. These facts may indicate that the differences between cases and controls in RANTES, IL-4, and IFN-γ are not primarily due to differences in atherosclerotic burden. It is not clear what causes the elevated levels of RANTES, IL-4, and IFN-γ. Medication with antiplatelet drugs[33–38] and statins[39–42] after ischemic stroke may affect concentrations of RANTES, IL-4, IFN-γ, and several other cytokines. However, from our results and from what has been described in previous studies, it seems unlikely that the elevated levels are explained solely by these treatments.

In our exploratory study, we cannot determine causal relationships. Prospective studies for establishing possible causal relationships and studies on the dynamics of cytokine levels after ischemic stroke are warranted. In this context, with elevated levels of RANTES, IL-4, and IFN-γ, one pathway that could be of interest for further investigation is the role of platelets. IFN-γ can contribute to platelet activation,[43,44] and platelets can modulate the secretion of IFN-γ in monocytes.[45] Activated platelets can release RANTES stored in α-granules.[46] RANTES in turn can activate basophils[47] which can release significant amounts of IL-4.[48] Whether these mechanisms are of relevance in ischemic stroke or not remains to be determined.

This study focused on comparing proinflammatory protein profiles in LAA stroke and cryptogenic stroke. In future studies, it would be of interest to examine these proteins in other main etiologic ischemic stroke subtypes, that is, cardioembolic (CE) and small artery occlusion (SAO) stroke. Interestingly, gene expression in peripheral blood has been profiled in 131 cases with cryptogenic stroke and compared with gene expression profiles in 149 ischemic stroke cases with known cause.[49] The latter cases were used to develop prediction models to distinguish CE from LAA stroke and to distinguish lacunar from non-lacunar causes of stroke. Prediction of cryptogenic strokes was then performed using gene expression in conjunction with infarct location, and causes of cryptogenic stroke were predicted to be cardioembolic in 58%, arterial (LAA) in 18%, and lacunar (SAO) in 12%.[49] It would thus be of interest to study proinflammatory plasma proteins such as RANTES, IL-4, and IFN-γ by a similar design in order to determine whether they separate lacunar from non-lacunar stroke and/or whether they could predict specific etiological subtypes within the cryptogenic group.

Strengths in the current study are inclusion of consecutive and well-characterized ischemic stroke patients as well as controls matched for age and sex, and strictly standardized blood sampling, performed both in the acute phase and after three months. The study also has limitations. In order to measure a panel of cytokines and chemokines, we used a bead array. As these arrays are less sensitive than conventional enzyme-linked immunosorbent assays (ELISA),[50] several analytes had a relatively high proportion of samples with undetectable concentrations, especially in controls. Furthermore, inter-assay coefficients of variation are relatively high for bead arrays. It has, however, been shown that inter-individual differences outweigh laboratory variation.[51] We aimed to minimize the influence of this variation by positioning both samples from each patient and the sample for the matched control in adjacent wells on the same microtiter plate. Furthermore, samples from the different stroke subtypes were relatively evenly distributed across the plates. Despite these precautions, we recognize that validation of the main findings with ELISA would be valuable. However, we were limited by plasma volume, and this was therefore not feasible. Although the cryptogenic group was defined based on an extensive clinical work-up, we recognize that it is most likely that some cases with cardioembolic causes were undetected. Moreover, the cryptogenic stroke group was stratified in low and high risk of atherosclerosis based on the FRS. As the investigations of the brain arteries also were performed with routine clinical work-up, we did not have direct measures of atherosclerotic burden such as stenosis of the internal carotid artery that was less than 50%, other atherosclerotic lesions, or intima-media thickness.

In conclusion, this study found that the plasma protein profiles separating patients from controls were similar in cryptogenic and LAA stroke. In both subtypes, RANTES, IL-4, and IFN-γ contributed the most to this separation, and the associations with ischemic stroke were independent of risk factors of atherosclerosis. Thus, the concentrations of RANTES, IL-4, and IFN-γ did not give guidance regarding stroke subtype. However, the finding that the levels of these proteins were still elevated three months post-stroke implies that they mainly reflect pre-stroke levels and could therefore have a role in the pathogenesis of ischemic stroke. When instead studying differences in protein profiles within the cryptogenic group, we found that the plasma proteins that contributed the most to the separation between cryptogenic stroke patients with low risk of atherosclerosis from those with high risk and from LAA stroke were eotaxin and MCP-1. Therefore, in patients with cryptogenic stroke, high levels of eotaxin and of MCP-1 may indicate atherosclerosis as an underlying pathology. Future studies to replicate these findings and to examine the roles of these proteins in atherosclerosis and in the pathogenesis of stroke are warranted.

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CONFLICTS OF INTEREST

HZ has served at scientific advisory boards for Denali, Roche Diagnostics, Wave, Samumed and CogRx, has given lectures in symposia sponsored by Fujirebio, Alzecure and Biogen, and is a co-founder of Brain Biomarker Solutions in Gothenburg AB, a GU Ventures-based platform company at the University of Gothenburg. KB has served as a consultant or at advisory boards for Abcam, Axon, Biogen, Lilly, MagQu, Novartis and Roche Diagnostics, and is a co-founder of Brain Biomarker Solutions in Gothenburg AB, which is a part of the GU Ventures Incubator Program. The other authors declare no financial or other conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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