Discovery of New Risk Markers for Ischemic Stroke Using a Novel Targeted Proteomics Chip

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Background and Purpose—Emerging technologies have made it possible to simultaneously evaluate a large number of circulating proteins as potential new stroke risk markers.

Methods—We explored associations between 85 cardiovascular proteins, assessed by a proteomics chip, and incident ischemic stroke in 2 independent cohorts of elderly (Prospective Investigation of the Vasculature in Uppsala Seniors [PIVUS]; n=977; 50% women, mean age=70.1 years, 71 fatal/nonfatal ischemic stroke events during 10.0 years; and Uppsala Longitudinal Study in Adult Men [ULSAM]; n=720, mean age=77.5 years, 75 ischemic stroke events during 9.5 years). The proteomics chip uses 2 antibodies for each protein and a polymerase chain reaction step to achieve a high-specific binding and the possibility to measure multiple proteins in parallel, but gives no absolute concentrations.

Results—In PIVUS, 16 proteins were related to incident ischemic stroke using a false discovery rate of 5%. Of these, N-terminal pro-B-type natriuretic peptide (P=0.0032), adrenomedullin (P=0.018), and eosinophil cationic protein (P=0.0071) were replicated in ULSAM after adjustment for established stroke risk factors. In predefined secondary meta-analyses of individual data, interleukin-27 subunit α, growth/differentiation factor 15, urokinase plasminogen activator surface receptor, tumor necrosis factor receptor superfamil member 6, macrophage colony-stimulating factor 1, and matrix metalloproteinase-7 were also potential risk markers for ischemic stroke after adjustment for multiple comparisons (P<0.0006). The addition of N-terminal pro-B-type natriuretic peptide, adrenomedullin, and eosinophil cationic protein to a model with established risk factors increased the C-statistic from 0.629 to 0.689 (P=0.001).

Conclusions—Our data suggest that large-scale proteomics analysis is a promising way of discovering novel biomarkers that could substantially improve the prediction of ischemic stroke. (Stroke. 2015;46:3340-3347. DOI: 10.1161/STROKEAHA.115.010829.)

Key Words: adrenomedullin ■ natriuretic peptide, brain ■ proteins ■ risk factors ■ stroke

Hypertension and atrial fibrillation are generally considered to be the most important modifiable risk factors for stroke, but in clinical practice, established cardiovascular risk factors, such as smoking, diabetes mellitus, high low-density lipoprotein cholesterol, and low high-density lipoprotein cholesterol, are also used to determine the stroke risk of a patient. Given that stroke is one of the leading causes of death and disability in the world, it is of the utmost importance to improve the identification of high risk individuals to prevent new onset stroke and lower the stroke burden on society.

In the past decades, several biomarkers, such as Lp(a)4,5 C-reactive protein,6 Lp-LPA2,8 the chemokine ligand CXCL12,7 intracellular adhesion molecule-1 (ICAM-1),8 homocysteine,9 brain natriuretic peptide,10 urinary albumin,10 and asymmetric dimethylarginine (ADMA) levels,11 have been put forward as novel risk markers for incident stroke, but to date none of these have made it to routine clinical practice. Emerging technologies provide new possibilities to discover new and clinically relevant biomarkers. It is today technically feasible to simultaneously measure 92 different proteins on an array chip based on the proximity extension assay technology. We recently designed such a chip-selecting protein previously shown to be related to atherosclerosis development or to other underlying mechanisms leading to cardiovascular disease in experimental or clinical studies.

In this study, we aimed to explore and validate the association of these 92 cardiovascular proteins and incident ischemic stroke in 2 independent community-based cohorts of elderly using a conservative statistical approach to take into account the multiple testing. To limit the risk of type II error, we also performed a predefined secondary meta-analysis based on individual data from the 2 cohorts.

Methods

PIVUS Study
Eligible subjects were 70 years of age and living in the city of Uppsala, Sweden. Subjects were chosen at random from the Total
Population Register. In total, 1016 individuals of 2025 invited took part in the investigation (50.1%) at age 70 years when also the blood sample for proteomics was collected.12 A follow-up of incident ischemic stroke was performed after 10 years, without any loss of follow-up. After exclusion of the 39 individuals with stroke before the baseline investigation in Prospective Investigation of the Vasculature in Uppsala Seniors (PIVUS), 71 of remaining 977 subjects experienced an ischemic stroke during a median follow-up of 10.0 years (range, 0.04–10.9 years), resulting in an incidence rate of 7.9 per 1000 person years at risk.

**ULSAM Study**

Uppsala Longitudinal Study in Adult Men (ULSAM) is a longitudinal population-based study including men born between 1920 and 1924 in Uppsala County, Sweden, being invited for the first time at age 50 years (n=2322).13 This study used the 77-year examination cycle as baseline because the proteomic chip was analyzed in samples collected at this time. Of 1398 invited men, 838 (60%) participated at this examination cycle. Of these, 75 were excluded because of missing blood samples for the proteomic analysis. Thus, 720 subjects were followed for a median of 9.5 years (range, 0.09–12.9 years) after exclusion of 43 subjects with stroke before the baseline investigation, without any loss of follow-up. During follow-up, 75 incident ischemic stroke events occurred (incidence rate of 13.0 per 1000 person years at risk).

We did not exclude any subjects in the analyses in any of the 2 cohorts if they have had other cardiovascular diseases, like myocardial infarction, heart failure, claudication, etc.

The median time from blood sample acquisition to diagnosis of ischemic stroke was 5.5 years (range, 0.1–11.1 years) when combining the 2 cohorts.

The Ethics Committee of Uppsala University approved the studies, and all subjects gave their informed prior consent.

All samples were collected in the morning after an overnight fast. The samples used for protein analysis were collected in EDTA plasma tubes and were kept on ice until spinning in a refrigerated centrifuge and then stored in −70°C until analysis. Two hundred microliters were used for the protein analysis.

Standard laboratory techniques were used to measure lipid variables and fasting blood glucose. Blood pressure was measured in the supine position after 15-min rest. A 12-lead ECG was performed for analysis of prevalent atrial fibrillation. Data on atrial fibrillation were collected and validated by medical records, the Swedish in-hospital registry and the Swedish cause of death registry.

**Proteomics**

The sample analyses were performed at the Clinical Biomarkers Facility, Science for Life Laboratory, Uppsala University, using the Olink Proseek Multiplex Cardiovascular 96x96 kit to simultaneously measure proteins in plasma by real-time polymerase chain reaction using the Fluidigm BioMark HD as described earlier.14,15 The resulting relative values, normalized protein expression units, obtained are log2 transformed for subsequent analysis. Mean intra-assay and interassay coefficients of variation were 8% and 12%, respectively. Further details about limit of detection (LOD), reproducibility, and validations are given at Olink webpage (http://www.oolink.com/products/proseek-multiplex/downloads/data-packages).

In the quality control and assurance process, we deleted proteins with a call rate <75%. Six such proteins were found in the PIVUS study (β-nerve growth factor, SIR2-like protein, interleukin-4 (IL-4), brain natriuretic peptide [BNP], nuclear factor-xB essential modulator, and melanin [ITGB1BP2]). In the ULSAM study, the corresponding number was 3 (ITGB1BP2, IL-4, and heat shock protein 27). Thus, data on 85 of the proteins were included in the statistical analyses. Values below LOD were replaced by LOD/216.

Seven subjects in the PIVUS study and 1 in the ULSAM study were removed because of a high number of proteins with measurements less than LOD. The data were adjusted for plate to remove any influence of drift in measurements between plates. Each protein was normalized by plate (by setting the mean to 0 and SD to 1 within each plate) and further by storage time (correction based on the observed values and predicted values from a spline model).

**Follow-Up**

During the follow-up periods, incident cases of fatal and nonfatal ischemic stroke (I63), including ischemic stroke, but not hemorrhagic stroke or transitory ischemic attacks were collected and validated by medical records, the Swedish in-hospital registry and the Swedish cause of death registry.

The vast majority of patients with stroke in Uppsala are treated at a highly specialized Stroke ward. The physicians at this ward, being trained in neurology and internal medicine, set the diagnosis based on clinical symptoms and a brain computed tomographic scan. Those diagnoses are then reported to the Swedish national registers. In addition, in the PIVUS study, and in a subset of the ULSAM cases, the diagnoses were validated by the use of the hospital computerized record system by one of the authors (L.L., who had worked several years at the Stroke ward).

**Statistical Analysis**

The proteomics data have first been log2 transformed to achieve normal distributions and thereafter transformed to a SD scale, so that the hazard ratios (HRs) should be comparable.

For the primary analysis, the PIVUS study was used as the discovery sample and ULSAM for replication. For discovery, a Cox proportional hazard analyses were performed for each of the 85 proteins adjusting for age and sex. The proteins showing a false discovery rate <0.05 were taken further to Cox proportional hazard analyses in the replication sample. At the replication step, 2 levels of adjustment were calculated, 1 with adjustment for age and sex only and 1 version adjusted for multiple risk factors (age, sex, low-density lipoprotein cholesterol and high-density lipoprotein cholesterol, systolic blood pressure, body mass index, diabetes mellitus, atrial fibrillation, and smoking). A nominal P value of <0.05 for the multiple-adjusted analysis was considered as a valid replication in ULSAM.

In predefined secondary meta-analysis using individual data, we merged the 2 data sets, and a Cox proportional hazard analyses were performed for each of the 85 proteins adjusting for age, sex, and study. The proteins showing a P value <0.00588 (Bonferroni-adjusted for 85 tests) were considered to be statistically significant. Thus, 170 separate regression models were used in the analysis, 2 for each protein. One of those being adjusted for age, sex, and study and the other also adjusted for traditional risk factors.

**Results**

Baseline characteristics of the study samples are given in Table 1.

**Discovery–Validation Approach**

When relating the 85 proteins to incident stroke one by one in PIVUS adjusting for age and sex, 16 proteins were related to incident stroke using a cutoff of false discovery rate <0.05. Results for all proteins in the discovery phase in PIVUS are given in Figure I in the online-only Data Supplement.

Of these, N-terminal pro-B-type natriuretic peptide (NT-pro-BNP; P=0.0032), adrenomedullin (P=0.018), and eosinophil cationic protein (ECP; P=0.0071) were replicated in ULSAM after adjustment for established stroke risk factors (Table 2; Figure II in the online-only Data Supplement). Further adjustments for statin and antiplatelet treatment did only marginally change the results (data not shown). Results for all proteins in the replication phase in ULSAM are given in Figure III in the online-only Data Supplement.
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Secondary Analyses

When the ULSAM and PIVUS cohorts were merged and used in the analysis, 9 of the proteins were related to incident stroke after adjustment for age, sex, and study using a Bonferroni correction for 85 tests ($P<0.000588$; NT-pro-BNP, IL-27 subunit $\alpha$ [IL27-$\alpha$], growth/differentiation factor 15 [GDF-15], adrenomedullin, urokinase plasminogen activator surface receptor [U-PAR], tumor necrosis factor receptor superfamily member 6 [FAS], macrophage colony-stimulating factor 1 [CSF-1], ECP, and matrix metalloproteinase-7 [MMP-7]; Table 3). These relationships were still highly significant after adjustment for traditional risk factors. Further adjustments for statin and antiplatelet treatment did only marginally change the results (data not shown). Results for all proteins in this merged analysis are given in Figure IV in the online-only Data Supplement.

When excluding ischemic stroke cases with known atrial fibrillation ($n=7$), the HR for NT-pro-BNP in the age- and sex-adjusted analysis was very similar (HR, 1.47; 95% confidence interval [CI], 1.22–1.82), whereas it was higher in the stroke cases with known atrial fibrillation (HR, 10.3; 95% CI, 2.00–53.4).

When the 162 subjects with known coronary heart disease at baseline were deleted from the analyses of the merged data sets, 126 ischemic stroke cases occurred during the follow-up period. The results were very similar compared with when the subjects with known coronary heart disease at baseline were included in the analyses, but in this case, ECP (HR, 1.40; 95% CI, 1.15–1.69; $P=0.00063$) and U-PAR (HR, 1.37; 95% CI, 1.14–1.65; $P=0.00095$) were no longer significant although their HRs were essentially unaltered. However, in this analysis,

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**Table 1. Basic Characteristics in the PIVUS ($n=977$) and ULSAM ($n=720$) Cohorts Given as Mean (SD) or Proportions**

<table>
<thead>
<tr>
<th>Variable</th>
<th>PIVUS</th>
<th>ULSAM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>70.1 (0.1)</td>
<td>77.5 (0.7)</td>
</tr>
<tr>
<td>Sex (% women)</td>
<td>51</td>
<td>0</td>
</tr>
<tr>
<td>Systolic blood pressure, mm Hg</td>
<td>149 (22)</td>
<td>150 (20)</td>
</tr>
<tr>
<td>Smoker (%)</td>
<td>11</td>
<td>8.2</td>
</tr>
<tr>
<td>Serum triglycerides, mmol/L</td>
<td>1.27 (0.60)</td>
<td>1.38 (0.69)</td>
</tr>
<tr>
<td>HDL-cholesterol, mmol/L</td>
<td>1.52 (0.42)</td>
<td>1.32 (0.33)</td>
</tr>
<tr>
<td>LDL-cholesterol, mmol/L</td>
<td>3.40 (0.88)</td>
<td>3.48 (0.87)</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>26.9 (4.3)</td>
<td>26.2 (3.5)</td>
</tr>
<tr>
<td>Diabetes mellitus, %</td>
<td>11</td>
<td>14</td>
</tr>
<tr>
<td>Waist circumference, cm</td>
<td>90.9 (11.4)</td>
<td>95.5 (10.4)</td>
</tr>
<tr>
<td>Atrial fibrillation, %</td>
<td>2.2</td>
<td>8.4</td>
</tr>
</tbody>
</table>

HDL indicates high-density lipoprotein; LDL, low-density lipoprotein; PIVUS, Prospective Investigation of the Vasculature in Uppsala Seniors; and ULSAM, Uppsala Longitudinal Study in Adult Men.

**Table 2. Analysis in the ULSAM Cohort Regarding the Risk of Stroke for the 16 Proteins That Showed a False Discovery Rate <0.05 in the PIVUS Study**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Age Adjusted Only</th>
<th></th>
<th></th>
<th>Multiple Adjusted</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR (95% CI)</td>
<td>$P$Value</td>
<td></td>
<td>HR (95% CI)</td>
<td>$P$Value</td>
</tr>
<tr>
<td>Adrenomedullin</td>
<td>1.33 (1.05–1.69)</td>
<td>0.018</td>
<td></td>
<td>1.39 (1.06–1.82)</td>
<td>0.018</td>
</tr>
<tr>
<td>Macrophage colony-stimulating factor 1</td>
<td>1.3 (1.02–1.65)</td>
<td>0.031</td>
<td></td>
<td>1.17 (0.90–1.52)</td>
<td>0.25</td>
</tr>
<tr>
<td>Eosinophil cationic protein</td>
<td>1.21 (0.93–1.57)</td>
<td>0.15</td>
<td></td>
<td>1.58 (1.13–2.2)</td>
<td>0.0071</td>
</tr>
<tr>
<td>Protein S100-A12</td>
<td>1.05 (0.83–1.32)</td>
<td>0.68</td>
<td></td>
<td>1.09 (0.84–1.41)</td>
<td>0.50</td>
</tr>
<tr>
<td>Tumor necrosis factor receptor superfamily member 6</td>
<td>1.24 (1.00–1.54)</td>
<td>0.046</td>
<td></td>
<td>1.18 (0.93–1.50)</td>
<td>0.17</td>
</tr>
<tr>
<td>Growth/differentiation factor 15</td>
<td>1.30 (1.04–1.63)</td>
<td>0.022</td>
<td></td>
<td>1.25 (0.96–1.62)</td>
<td>0.10</td>
</tr>
<tr>
<td>Heparin-binding EGF-like growth factor</td>
<td>1.00 (0.80–1.25)</td>
<td>0.98</td>
<td></td>
<td>0.99 (0.77–1.28)</td>
<td>0.94</td>
</tr>
<tr>
<td>Monocyte chemotactic protein 1</td>
<td>0.98 (0.78–1.24)</td>
<td>0.87</td>
<td></td>
<td>1.10 (0.85–1.43)</td>
<td>0.46</td>
</tr>
<tr>
<td>Matrix metalloproteinase-12</td>
<td>1.12 (0.89–1.41)</td>
<td>0.33</td>
<td></td>
<td>1.13 (0.87–1.46)</td>
<td>0.35</td>
</tr>
<tr>
<td>Matrix metalloproteinase-7</td>
<td>1.20 (0.95–1.52)</td>
<td>0.12</td>
<td></td>
<td>1.14 (0.87–1.50)</td>
<td>0.33</td>
</tr>
<tr>
<td>N-terminal pro-B-type natriuretic peptide</td>
<td>1.72 (1.33–2.22)</td>
<td>0.000035</td>
<td></td>
<td>1.52 (1.15–2.02)</td>
<td>0.0032</td>
</tr>
<tr>
<td>Proteinase-activated receptor 1</td>
<td>1.13 (0.89–1.43)</td>
<td>0.32</td>
<td></td>
<td>1.07 (0.83–1.40)</td>
<td>0.58</td>
</tr>
<tr>
<td>Spondin-1</td>
<td>1.19 (0.94–1.50)</td>
<td>0.15</td>
<td></td>
<td>1.14 (0.88–1.48)</td>
<td>0.31</td>
</tr>
<tr>
<td>TNF receptor 1</td>
<td>1.20 (0.95–1.53)</td>
<td>0.13</td>
<td></td>
<td>1.16 (0.88–1.52)</td>
<td>0.28</td>
</tr>
<tr>
<td>TNF-related apoptosis-inducing ligand receptor 2</td>
<td>1.27 (1.00–1.60)</td>
<td>0.048</td>
<td></td>
<td>1.21 (0.92–1.58)</td>
<td>0.17</td>
</tr>
<tr>
<td>Urokinase plasminogen activator surface receptor</td>
<td>1.21 (0.95–1.54)</td>
<td>0.12</td>
<td></td>
<td>1.29 (0.98–1.70)</td>
<td>0.070</td>
</tr>
</tbody>
</table>

HR and 95% CI are given for 2 levels of adjustments: age only (men only in ULSAM) and multiple cardiovascular risk factors (age, low-density lipoprotein cholesterol and high-density lipoprotein cholesterol, systolic blood pressure, body mass index, diabetes mellitus, atrial fibrillation, and smoking). CI indicates confidence interval; HR, hazard ratio; PIVUS, Prospective Investigation of the Vasculature in Uppsala Seniors; TNF, tumor necrosis factor; and ULSAM, Uppsala Longitudinal Study in Adult Men.
2 proteins not being significant in the former analysis showed a P value below the Bonferroni-adjusted level. Tumor necrosis factor receptor 1 (TNFR1), and endothelial cell–specific molecule 1 (Table I in the online-only Data Supplement).

**Prediction of Ischemic Stroke**

When the ULSAM and PIVUS cohorts were merged and used to evaluate whether the 3 proteins identified in the discovery/replication phase (NT-pro-BNP, adrenomedullin, and ECP) would add to the predictive power beyond the traditional risk factors (age, sex, low-density lipoprotein cholesterol and high-density lipoprotein cholesterol, systolic blood pressure, body mass index), diabetes mellitus, atrial fibrillation, and smoking), the addition of these 3 proteins increased C-statistics significantly from 0.629 (95% CI, 0.582–0.676) for the base model to 0.663 (95% CI, 0.616–0.709; P=0.030) for the addition of NT-pro-BNP, 0.657 (95% CI, 0.610–0.704; P=0.052) for the addition of adrenomedullin, 0.656 (95% CI, 0.610–0.709; P=0.073) for the addition of ECP, and 0.689 (95% CI, 0.641–0.737; P=0.0012) for the simultaneous addition of all 3 proteins.

**Discussion**

In this study, a novel state-of-the-art targeted proteomics chip was used to investigate the associations between a large number of circulating cardiovascular proteins and the incidence of ischemic stroke. Using a conservative discovery–replication approach in 2 independent cohorts of elderly, NT-pro-BNP, ECP, and adrenomedullin were robustly associated with the risk of ischemic stroke incidence independently of established cardiovascular risk factors and prevalent atrial fibrillation. Importantly, the incorporation of the 3 proteins to a model with established risk factors improved the risk stratification for ischemic stroke considerably, as evidenced by a substantial increase in the C-statistic.

Secondary meta-analyses using individual data suggested that also GDF-15, IL-27-α, U-PAR, FAS, CSF-1, and MMP-7 might be of importance about ischemic stroke. NT-pro-BNP, adrenomedullin, and GDF-15 have previously been suggested to be independent risk factors for stroke in the primary preventive setting in the community, whereas ECP, IL-27-α, U-PAR, FAS, CSF-1, and MMP-7 are novel ischemic stroke risk markers.

**N-Terminal Pro-B-Type Natriuretic Peptide**

NT-pro-BNP is the N-terminal part of the propeptide of BNP, a natriuretic peptide secreted from the myocardial ventricles in response to stress and volume overload. Clinically, NT-pro-BNP is used as markers for heart failure, but those proteins have also been identified as risk factors for all-cause mortality, cardiovascular mortality, myocardial infarction, and atrial fibrillation.

However, data on BNP and NT-pro-BNP about risk of stroke in the community are sparse.

In stroke patients with atrial fibrillation, BNP levels were associated with recurrent stroke, and BNP or NT-pro-BNP levels have been suggested to differentiate cardioembolic stroke from other stroke subtypes. Recently, BNP and NT-pro-BNP were both found to be associated with incident stroke in a community-based sample free of stroke at baseline.

In this study, NT-pro-BNP was the protein on the chip by far being most closely related to stroke risk, despite adjustment for atrial fibrillation at baseline. When we in an additional analysis deleted the 70 individuals with atrial fibrillation at baseline, the age- and sex-adjusted HR for NT-pro-BNP was essentially unaltered, suggesting that NT-pro-BNP might be linked to stroke risk by other mechanisms than by atrial fibrillation. Still, we cannot completely rule out residual confounding by undetected paroxysmal atrial fibrillation.

When we divided the ischemic stroke cases in those with and without known atrial fibrillation, the HR for NT-pro-BNP...
was substantially higher for the cases with atrial fibrillation than in those without. However, the number of ischemic stroke cases with known atrial fibrillation was limited, and therefore the results of this tertiary analysis must be taken with caution.

**Eosinophil Cationic Protein**

ECP is a protein liberated by eosinophil cells on activation. Although elevated levels most often have been associated with allergic reactions, increased levels of ECP have also been described in patients with coronary heart disease. Eosinophils and eosinophil granula proteins, such as ECP, have been shown to be able to induce a prothrombotic and proinflammatory endothelial phenotype and could thereby well be involved in the stroke process.

In this study, ECP was identified as a risk factor for stroke in the discovery–replication approach following adjustment for cardiovascular risk factors and also showed a significant P value following Bonferroni adjustment following adjustment for cardiovascular risk factors.

**Adrenomedullin**

The primary action associated with adrenomedullin is vasodilation, but also other functions, such as angiogenesis, protection from oxidative stress, and hypoxic injury, have been linked to this hormone. However, adrenomedullin, or its precursor, midregional proadrenomedullin, levels have been associated in a positive fashion to atherosclerosis and to prevalence stroke in cross-sectional studies as well as to incident ischemic stroke in this study. This might seem contra intuitive given that the primary action of adrenomedullin would be protective against atherosclerosis and cardiovascular disease. However, as seen for several other biomarkers, the increased adrenomedullin levels could be a mean to compensate for an ongoing pathological process. Consequently, adrenomedullin as a biomarker would be related to stroke in a positive fashion.

**Interleukin-27**

IL-27-α is a member of the IL-12 family of cytokines. The findings from recent years identify IL-27 as a critical immunoregulatory cytokine, especially for T cells, whereas some controversy exists about the view of IL-27 as a classical silencer of inflammation, as reviewed by Bosmann et al. There are some experimental evidence that support a role of IL-27-α in stroke pathophysiology; Hirase et al demonstrated that mice lacking the IL-27 receptor were more susceptible to atherosclerosis compared with the wild-type because of enhanced accumulation and activation of macrophages in arterial walls.

**Urokinase-Type Plasminogen Activator Surface Receptor**

U-PA is a protease enzyme in the fibrinolytic system, and U-PA binding to its receptor, U-PAR, leads to plasmin generation and plasmin-mediated proteolysis. However, U-PA/U-PAR has recently been shown also to play other roles, such as being involved in cell migration, differentiation, and matrix degradation, as reviewed by Fuhrman. U-PA is expressed by macrophages within human atherosclerotic lesions. Elevated plasma levels of U-PA have been found in patients with unstable angina and were related to plaque plus media and external elastic membrane areas.

**Growth/Differentiation Factor-15**

GDF-15 is a member of the transforming growth factor-β cytokine superfamily, and its expression is increased in myocardial and vascular cells on oxidative stress and inflammation. Elevated levels of GDF-15 have been related to increased risk of both cardiovascular and noncardiovascular mortality. GDF-15 measured by an ELISA technique has previously been reported to be a risk factor for stroke in the ULSAM study when measured at age 70 years. In this study, GDF-15 levels, assessed by the proteomics chip at age 77 years, predicted stroke in age-adjusted analyses, but was no longer statistically significant after adjustment for cardiovascular risk factors (P=0.054). However, GDF-15 was clearly significant in the meta-analysis using both samples.

**Tumor Necrosis Factor Receptor Superfamily Member 6**

The FAS receptor, also known as apoptosis antigen 1 (APO-1 or APT), or TNFR superfamily member 6 (TNFRSF6) is a receptor involved in apoptosis, where FAS forms a death-inducing signaling complex on ligand binding. Soluble FAS ligand has been associated with atherosclerosis in man. FAS increases in serum after acute ischemic stroke, but this is the first study that FAS levels are associated with incident ischemic stroke.

**Macrophage Colony-Stimulating Factor 1**

CSF-1 is a cytokine and a hematopoietic growth factor involved in the proliferation, differentiation, and survival of monocytes, macrophages, and bone marrow progenitor cells. CSF-1 plays an important role in atherosclerosis formation, as reviewed by Andrés et al. Elevated levels of CSF-1 have previously been reported in patients with an old stroke, but this is the first study describing a link with incident ischemic stroke.

**Matrix Metalloproteinase-7**

MMP-7 belongs to the MMP family with the primary action to degrade extracellular matrix, including casein, type I, II, IV, and V gelatins, fibronectin, and proteoglycan. MMP-7 has been linked to human atherosclerosis and to incident cardiovascular disease, but this is the first study linking MMP-7 to incident ischemic stroke.

**Ischemic Stroke and Atherosclerosis**

A recently published cross-sectional analysis in the PIVUS study on carotid artery atherosclerosis and the same proteomics chip using in the present study disclosed 7 of the proteins to be significantly related to the number of carotid arteries affected by plaques in age- and sex-adjusted models (osteoprotegrin, T-cell immunoglobulin and mucin domain-1, GDF-15, MMP-12, growth hormone, TNFSF14, and renin). To note is that of the 16 proteins that showed a false discovery rate <0.05 about incident ischemic stroke in the PIVUS sample in this study, only MMP-12, osteoprotegrin, and GDF-15 were identified to be related to carotid artery atherosclerosis.
Clinical Implications
In our 2 cohorts of elderly, the C-statistics analyses clearly suggest that the addition of NT-pro-BNP, ECP, and adrenomedullin to a model with established stroke risk factors substantially improves the ability to discriminate individuals with a higher risk, from those with a lower risk. Given the growing global burden of stroke, \(^1\) an improved risk prediction will be increasingly important to lower the incidence of new onset stroke. This may be particularly important in the elderly, where the relative risks associated with the established cardiovascular risk factors have been shown to be diminished with higher age. \(^2\) Although our data are promising, it should be noted that there is currently no evidence that reducing the circulating levels of these proteins will reduce the risk of ischemic stroke. Thus, the clinical utility of our findings is still uncertain, and further studies are warranted.

Strengths and Limitations
The strengths of the study include the longitudinal study design with up to 10 years of follow-up, the detailed characterization of study participants, the use of a novel state-of-the-art proteomics chip, and the stringent statistical analyses plan with replication of findings in an independent cohort. However, using a discovery–replication design in our primary analysis, we had a somewhat limited number of ischemic stroke events in each of the cohorts, and consequently, a limited power that may have underestimated the number of independent predictors of ischemic stroke. We therefore also performed a predefined secondary exploratory approach increasing the power by merging the 2 samples into a meta-analysis based on individual data. In this case, we used a conservative approach only reporting the proteins being significant after Bonferroni adjustment. It should however be pointed out that that the additional proteins identified by the secondary approach have to be replicated in other samples to be regarded as valid.

The present findings were obtained in the elderly in a geographically defined part of Sweden, and the findings have therefore to be validated internationally in younger subjects and in other ethnic groups.

The proteins on the chip were a selection from a large number of proteins that previously have been reported in the literature to be of interest about cardiovascular disease or atherosclerosis in human or experimental studies. The final selection process was based on both the availability of proper antibodies and the concentration limits of the analytes. Thus, the proteomics chip in its present form is not ideal but should be regarded as a prototype for further development and biomarker discovery. Another limitation of the proteomics chip is that no absolute levels of the proteins could be obtained to compare between studies or defining relevant cutoff limits.

In this study, we only used ischemic stroke cases. We did only have a limited number of hemorrhagic stroke cases, so a comparison between ischemic and hemorrhagic stroke could not be performed in a powerful way.

Conclusions
Our data confirm and extend the notion that large-scale proteomics analysis is a promising way of discovering novel biomarkers that could substantially improve the prediction of stroke.

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Disclosures
Dr Siegbahn received consulting fees from Olink. Dr Lindahl has served as a Consultant in Roche Diagnostics, bioMérieux Clinical Diagnostics, Philips Healthcare, Thermo-Fischer, and Fiomi Diagnostics. Dr Sundström participated in Expert panel at Itrim. The other authors report no conflicts.

References


Discovery of New Risk Markers for Ischemic Stroke Using a Novel Targeted Proteomics Chip

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DISCOVERY OF NEW RISK MARKERS FOR ISCHEMIC STROKE USING A NOVEL TARGETED PROTEOMICS CHIP

Supplementary Table, Figures and Figure Legends
Supplementary Table I. Analysis regarding risk of stroke for 85 different proteins using a meta-analysis of individual data from the PIVUS and ULSAM studies when excluding subjects with coronary heart disease at baseline. Only the proteins with an age-adjusted p-value < 0.000588 (Bonferroni adjustment) are shown. Hazard ratios (HR) and 95% confidence limits (CI) are given for two levels of adjustments; age, sex and study and multiple cardiovascular risk factors (age, sex, study, LDL- and HDL-cholesterol, systolic blood pressure, BMI, diabetes, atrial fibrillation and smoking).

<table>
<thead>
<tr>
<th>Protein</th>
<th>Age adjusted only</th>
<th>Multiple adjusted</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-terminal pro-B-type natriuretic peptide (NT-pro-BNP)</td>
<td>1.44 (1.23, 1.7)</td>
<td>1.36 (1.15, 1.62)</td>
</tr>
<tr>
<td>Tumor necrosis factor receptor superfamily member 6 (FAS)</td>
<td>1.37 (1.19, 1.57)</td>
<td>1.31 (1.13, 1.51)</td>
</tr>
<tr>
<td>Adrenomedullin (AM)</td>
<td>1.51 (1.25, 1.83)</td>
<td>1.47 (1.2, 1.8)</td>
</tr>
<tr>
<td>Growth/differentiation factor 15 (GDF-15)</td>
<td>1.44 (1.2, 1.72)</td>
<td>1.34 (1.1, 1.64)</td>
</tr>
<tr>
<td>Macrophage colony-stimulating factor 1 (CSF-1)</td>
<td>1.44 (1.2, 1.74)</td>
<td>1.35 (1.11, 1.63)</td>
</tr>
<tr>
<td>Tumor necrosis factor receptor 1 (TNF-R1)</td>
<td>1.4 (1.17, 1.67)</td>
<td>1.35 (1.12, 1.62)</td>
</tr>
<tr>
<td>Matrix metalloproteinase-7 (MMP-7)</td>
<td>1.3 (1.12, 1.5)</td>
<td>1.22 (1.04, 1.43)</td>
</tr>
<tr>
<td>Interleukin-27 subunit alpha (IL27-A)</td>
<td>1.38 (1.15, 1.65)</td>
<td>1.35 (1.11, 1.63)</td>
</tr>
<tr>
<td>Endothelial cell-specific molecule 1 (ESM-1)</td>
<td>1.37 (1.15, 1.65)</td>
<td>1.43 (1.18, 1.72)</td>
</tr>
</tbody>
</table>
Supplementary Figure I. Relationships between 85 proteins and incident stroke in the PIVUS study (discovery step). Hazard ratio (HR) is given together with 95%CI for the age and sex-adjusted analyses.
Supplementary Figure II. Distributions for the three proteins identified in the discovery/replication phase (NT-pro-BNP, Adrenomedullin and ECP) are given in those with and without incident ischemic stroke separately. The box plots display the median, the 25th and 75th percentiles. Observe that no absolute levels are given and that the proteins are given on a SD-scale.
Supplementary Figure III. Relationships between 85 proteins and incident stroke in the ULSAM study (replication step). Hazard ratio (HR) is given together with 95%CI for the age and sex-adjusted analyses.
Supplementary Figure IV. Relationships between 85 proteins and incident stroke in merged analysis of the PIVUS and ULSAM studies. Hazard ratio (HR) is given together with 95%CI for the age, sex and study-adjusted analyses.
Discovery of New Risk Markers for Ischemic Stroke Using a Novel Targeted Proteomics Chip

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Abstract

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