Low Levels of Antibodies Against Phosphorylcholine Predict Development of Stroke in a Population-Based Study From Northern Sweden

Roland Fiskesund, MD; Birgitta Stegmayr, MD, PhD; Göran Hallmans, MD, PhD; Max Vikström; Lars Weinehall, MD, PhD; Ulf de Faire, MD, PhD; Johan Frostegård, MD, PhD

Background and Purpose—Natural immunoglobulin M antibodies specific for phosphorylcholine (anti-PC) have been implicated in atherosclerosis. We have shown previously that high levels of anti-PC predict a slower progression of atherosclerosis in humans and that low levels of anti-PC are associated with higher risk for cardiovascular disease. Here we determine the association between anti-PC and the incidence of stroke.

Methods—Using a nested case control study design, we examined 227 incident cases (125 men and 102 women) of first-time stroke and 455 age- and sex-matched controls identified during a 13-year time period (1985 to 1999) within the population-based cohorts of the Västerbotten Intervention Project (VIP) and the World Health Organization Monitoring Trends and Determinants in Cardiovascular Disease (WHO MONICA) project in Northern Sweden. Odds ratios of stroke with 95% CIs with adjustments for age, gender, smoking, serum cholesterol, diabetes, body mass index, and blood pressure were determined. Anti-PC levels were measured using ELISA.

Results—A significant association between low levels of anti-PC at baseline and incident stroke was seen for the whole group of anti-PC levels below the 30th percentile (multivariately adjusted odds ratio, 1.62; CI, 1.11 to 2.35). Analyses of gender-specific associations indicated fairly strong associations for females, especially at the lowest 30th percentile (multivariately adjusted odds ratio, 2.65; CI, 1.41 to 4.95). No associations were noted for men.

Conclusion—Low anti-PC is a novel independent risk marker for development of stroke. Measurements of anti-PC could be used to identify immunodeficient subjects at an increased risk for stroke. The possibility that such subjects might be targets for novel modes of treatment such as immunotherapies deserves further investigation. (Stroke. 2010;41:607-612.)

Key Words: immunology ■ inflammation

Atherosclerosis is the major underlying cause of cardiovascular disease (CVD), such as stroke and myocardial infarction, and can be characterized as an inflammatory disease in which activated immune competent cells are abundant. Oxidized or enzymatically modified forms of low-density lipoprotein are abundant in lesions and activate monocytes, endothelial cells, and T cells and may be a major factor causing the inflammation in atherosclerosis. Traditional risk factors including age, male sex, smoking, hypertension, hyperlipidemia and dyslipidemia, and diabetes do not reflect the inflammatory and immunologic components of atherosclerosis and CVD. Therefore, C-reactive protein, platelet-activating factor–acyethylhydrolase (LP-PLA2), and antibodies against heat shock proteins, among others, have been put forward as possible new risk markers.

The phosphorylcholine antibody of immunoglobulin M (IgM) subclass (anti-PC) is a natural antibody produced by a subset of CD5+ B cells independent of external antigen stimuli, as demonstrated by their presence in germ-free mice. PC is a part of phosphatidylcholine, a ubiquitous phospholipid found on cell membranes and on low-density lipoprotein. It is readily oxidized, and its oxidized form is one of the hallmarks of apoptotic cells and oxidized low-density lipoprotein. During oxidation, platelet-activating factor–like phospholipids cause much of the proinflammatory effects of oxidized low-density lipoprotein, and PC is essential for these effects. Anti-PC does not bind to native phosphatidylcholine. Since we first demonstrated that high levels of anti-PC predict a slower progression of carotid atherosclerosis in humans, we have consistently shown that low levels of anti-PC are associated with CVD. However, stroke as the primary end point has not been studied. In the current study, we now report that low anti-PC predicts increased risk for stroke. The implications of this finding are discussed.
Subjects and Methods

We used a nested case-control study design nested in 2 population-based cohorts: the Västerbotten Intervention Project (VIP) and the WHO MONICA project in Northern Sweden. In the pasts, these cohorts and this study design have been used to explore risk factors for CVD, and here, we are using it to assess the validity of anti-PC as a risk marker for future stroke. The cohorts are population based.21

The MONICA project was part of the worldwide WHO initiative to study trends in CVD. Every fourth year, 2000 to 2500 persons living in the 2 northernmost counties of Sweden, 25 to 74 years of age, were invited to a health examination, during which height, weight, blood pressure, smoking habits, serum cholesterol, and lifestyle habits were recorded.22 Participants were also encouraged to donate blood for future research, stored as heparin–plasma in the Northern Sweden Medical Research Bank.23 In this study, we used data from the Northern Sweden MONICA project for the years 1985 through 1999.

VIP is an ongoing project in the Swedish county of Västerbotten. It was initiated in 1985 and was originally intended as a health promotion program for the population in one county (Västerbotten; ~250,000 inhabitants). Every year, all persons reaching the ages of 30, 40, 50, and 60 are invited to their local health centers for a routine screening. The questionnaire for VIP is very similar to the one used in the MONICA project. All participants are asked to donate blood samples, which are stored in the above-mentioned biobank. By December 2002, a total of 74,000 unique people had attended health examinations within the framework of the project.

The participation rate was 60% for VIP and 77.2% for MONICA. Previous publications on nonparticipants have shown that nonparticipants in VIP are quite similar to the participants from a socioeconomic standpoint, indicating a marginal social selection bias.24

Measurements of Biomedical Factors

Smoking habits were characterized as daily smokers, 2 ex-smokers, and nonsmokers. Blood pressure was recorded after 5 minutes of rest in supine position. Hypertension was defined as systolic blood pressure ≥140 mm Hg and/or diastolic blood pressure ≥90 mm Hg, and/or antihypertensive drug treatment. Samples for lipid measurements were obtained after a minimum of 4 hours of fasting. Total serum cholesterol was measured using a bench top analyzer (Reflotron; Boehringer Mannheim GmbH; Diagnostica, Germany) or by an enzymatic method (Boehringer Mannheim GmbH; Diagnostica, Germany). Hypercholesterolemia was defined as serum cholesterol ≥5.0 mmol/L. Weight was measured in light indoor clothing and recorded to the nearest 0.5 kg, and height was measured to the nearest centimeter without shoes. Body mass index was calculated as weight (kg)/height2 (m2). History of diabetes was obtained from the questionnaire question: “Do you have diabetes?”

Case Definition and Selection of Controls

The WHO criteria define stroke as rapidly developing clinical signs of focal (or global) disturbance of cerebral function lasting ≥24 hours (unless interrupted by surgery or death) with no apparent cause other than vascular origin. This definition excludes transient ischemic attacks, subdural hemorrhage, traumatic intracerebral hemorrhage, and lesions caused by brain tumors. All suspected acute strokes in Northern Sweden are validated against strict WHO MONICA criteria24 and are recorded in a national stroke register. In the current study, we used the stroke registry to identify first-ever stroke among persons from the combined population-based cohorts of VIP and MONICA. Using the Swedish cancer and acute myocardial infarction registries, cases with previous acute myocardial infarction or cancer were excluded from the analysis. For every stroke case, 2 controls were selected from the VIP MONICA cohorts and matched for age, sex, geographic localization, and follow-up time. Controls were also required to be free from previous acute myocardial infarction and cancer. For more information, please see to previous publications from this cohort.25,26 In total, 227 first-ever strokes (125 men and 102 women) were recruited as well as an additional 455 controls, 29 to 74 years of age at baseline. The

| Table 1. Baseline Characteristics of Subjects With Stroke and Controls |
|-----------------|-----------------|-----------------|-----------------|
| Age (years)     | Cases Values    | Controls Values | P Value         |
| Male sex (%)    | 57              | 57              | NA              |
| Daily smokers (%) | 25              | 19.6            | 0.0835          |
| Body mass index | 26.9±4.2        | 25.9±3.5        | 0.0007          |
| Serum cholesterol (mmol/L) | 6.4±1.3        | 6.2±1.2        | 0.0463          |
| Hypertension (≥140/90 mm Hg), (%) | 67              | 45              | <0.0001         |
| Systolic blood pressure (mm Hg) | 143.8±20       | 135.7±17.9     | <0.0001         |
| Diastolic blood pressure (mm Hg) | 88.7±10.4      | 84.8±9.0       | <0.0001         |
| Anti-PC (U/ml)  | 50.9 (30–82.2)  | 52.9 (34–84.5)  | 0.0989          |
| Diabetes (%)    | 5.5             | 1.9             | 0.0163          |

*Data are presented as mean±SD, median and interquartile range, or percentage.

average age was 55 at inclusion, and the mean follow-up time was 5.6 years.

Ethical Permission

Ethical permission for the study was issued by the ethics committee at Umeå University, and the computerized patient database was approved by the Swedish National Computer Data Inspection Board. Informed consent was obtained at the time of the health surveys, and if a stroke occurred, informed consent was obtained from patients and the matched control subjects.

Antibody Determinations

Detection of IgM anti-PC antibodies was performed with an ELISA using Athera CVDefine kit (Athera CVDefine; Athera Biotechnologies AB). The assay is based on PCs covalently linked to BSA coated onto 96-well microtiter plates. The assay was performed in accordance with manufacturer recommendations. The detection limit of Athera anti-PC was 0.5 U/mL, as estimated from dilutions of the calibrator containing 6.25 U/mL of IgM anti-PC. The coefficients of variation for the samples were <7%.

Statistical Analysis

Antibody levels were dichotomized or determined as continuous variables as indicated. We calculated percentiles based on data from the whole study group. Wilcoxon rank sum tests or t tests were applied depending on the distribution of data. The association between antibodies and incident stroke were determined by conditional logistic regression models with calculation of odds ratios and 95% CIs. Age, gender, and geography were matched for by the design of the study. Adjustments were made for possible confounders including smoking habits, body mass index, diabetes, hypercholesterolemia (serum cholesterol ≥5.0 mmol/L), and hypertension (≥140/90 mm Hg systolic/diastolic) as indicated. A 2-tailed P value <0.05 was considered significant. Receiver operating characteristic curves and the area under those curves for stroke prediction with and without anti-PC were calculated. SAS was used for statistical analyses (release 9.1; SAS Institute Inc.).

Results

Demographic, clinical, metabolic, and other characteristics of the subjects at the time of enrollment, presented for the whole cohort, are summarized in Table 1. The VIP cohort represents 92% and MONICA 8% of the subjects. Because of matching
criteria at inclusion, there were negligible differences in age and no differences in gender between cases and controls.

The mean age at inclusion was 55 years (29 to 74 years) for the whole cohort, and 57% were males. The average time until stroke occurred was 5.6 years. Subjects who developed stroke more often had hypertension (67% versus 45%) and were more often smokers (25% versus 19.6%). There were small differences in the baseline characteristics when data for the combined cohort were compared with the data of the 2 subcohorts.

The group of subjects experiencing hemorrhagic stroke was relatively small, whereas the majority of cases were attributable to ischemic stroke (Table 2). Therefore, statistical analysis is presented on the total cohort of stroke cases and not of subgroups based on type of stroke because the study is underpowered to get reliable results from the hemorrhagic subgroup. However, excluding the hemorrhagic strokes from the analysis did not impact the results (data not shown).

### Table 2. Subdivision of the Stroke Cases

<table>
<thead>
<tr>
<th></th>
<th>Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemorrhagic stroke</td>
<td>27</td>
<td>14</td>
</tr>
<tr>
<td>Ischemic stroke</td>
<td>125</td>
<td>102</td>
</tr>
<tr>
<td>Unspecified stroke</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Sum</td>
<td>155</td>
<td>117</td>
</tr>
</tbody>
</table>

### IgM Anti-PC and Risk of Stroke

Median levels of IgM anti-PC levels did not differ significantly between cases and controls. However, when analyzing relative risks for stroke at various cut-off levels for anti-PC levels, significance was reached at the 30th and 20th percentiles (Table 3).

The most apparent relationship between anti-PC levels and risk for stroke was noted below the 30th percentile (multivariately adjusted relative risk, 1.62; CI, 1.11 to 2.35). When gender-specific analyses were performed, significant relative risks for stroke were seen for women at levels of IgM anti-PC less than or equal to 0.684, a significant increase in the maximum likelihood ratio test (Tables 3–5).

#### Discussion

Here we report that low levels of IgM anti-PC confer an increased risk for stroke in a population-based nested case-control study from Northern Sweden, which was designed to study first-ever incident strokes. This association was independent of and unaffected by other risk factors, including smoking habits, body mass index, diabetes, hypercholesterolemia, and hypertension.

This finding confirms and extends our previous reports regarding an inverse relationship between anti-PC and atherosclerosis or CVD, and this is the first time we studied the role of IgM anti-PC in a study specifically designed to study incident stroke. The first of our reports on IgM anti-PC was from a relatively small study on hypertensive patients in which particularly high levels of anti-PC were associated with decreased atherosclerosis development (assessed by repetitive ultrasound measurements of the carotids) after 5 years. Low anti-PC IgM levels were nonsignificantly associated with increased atherosclerosis development.

To further assess whether low levels of anti-PC could be associated with increased risk for atherosclerosis-related complications such as ischemic stroke and myocardial infarction, we reported, in 2 population-based nested case-control studies (one from Southern Sweden [Malmö Diet Cancer Study] and one from Northern Sweden [FIA-2 from VIP/MONICA-cohorts]) that low levels of IgM anti-PC predict increased risk of CVD. In a subgroup analysis of the Malmö Diet Cancer study, low anti-PC levels came out as a significant predictor for ischemic stroke, with a relative risk as high as approaching 4 among men. However, there were

#### Table 3. Association Between Low Anti-PC Levels and Risk of Stroke in General

<table>
<thead>
<tr>
<th>Percentiles</th>
<th>Anti-PC Levels (Men and Women)</th>
<th>No. of Cases = 227</th>
<th>Crude</th>
<th>OR 95% CI</th>
<th>Adjusted for Confounders* No. of Cases = 227</th>
<th>OR 95% CI</th>
<th>ROC (AUC Without Anti-PC)</th>
<th>ROC (AUC With Anti-PC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>51.910</td>
<td>120</td>
<td>1.215</td>
<td>0.878–1.680</td>
<td>1.228</td>
<td>0.872–1.729</td>
<td>0.6272</td>
<td>0.6295</td>
</tr>
<tr>
<td>40</td>
<td>44.278</td>
<td>98</td>
<td>1.235</td>
<td>0.882–1.727</td>
<td>1.292</td>
<td>0.903–1.849</td>
<td>0.6272</td>
<td>0.6281</td>
</tr>
<tr>
<td>30</td>
<td>35.989</td>
<td>80</td>
<td>1.440</td>
<td>1.017–2.038</td>
<td>1.617</td>
<td>1.112–2.351</td>
<td>0.6272</td>
<td>0.6355</td>
</tr>
<tr>
<td>20</td>
<td>29.402</td>
<td>54</td>
<td>1.490</td>
<td>1.004–2.212</td>
<td>1.603</td>
<td>1.049–2.452</td>
<td>0.6272</td>
<td>0.6336</td>
</tr>
<tr>
<td>10</td>
<td>22.273</td>
<td>24</td>
<td>1.147</td>
<td>0.674–1.954</td>
<td>1.312</td>
<td>0.745–2.311</td>
<td>0.6272</td>
<td>0.6261</td>
</tr>
<tr>
<td>4</td>
<td>17</td>
<td>12</td>
<td>1.160</td>
<td>0.749–3.418</td>
<td>1.904</td>
<td>0.822–4.411</td>
<td>0.6272</td>
<td>0.6305</td>
</tr>
</tbody>
</table>

*Smoking habits, body mass index (>28.14), diabetes, hypercholesterolemia (serum cholesterol ≥5.0 mmol/L), and hypertension (≥140/90 mm Hg systolic/diastolic).

OR indicates odds ratio; AUC, area under curve; ROC, receiver operating characteristic curve.
too few females in this study to provide reliable data on women. In the FIA-2 study from Northern Sweden, only incident myocardial infarctions were studied. The results of the Malmo Diet Cancer study, in which stroke was included as a subgroup analysis, differ from the present study because here we only noted low anti-PC to be related to an increased risk in women, whereas in the previous report, this association was noted in men only. Our current finding was therefore a bit surprising. However, there could be several explanations for this discrepancy (power issues, differences in population structure, age distributions, etc). First, in the present study, stroke was the major objective. Second, in the Malmo study, the number of women was relatively low, providing analyses with low power. Further on, in addition to the geographical, genetic, socioeconomic, and dietary differences between the cohorts, the most striking difference is the age structures of the studies. The subjects in the current study are an average of 5 years younger than those included in the Malmo Diet Cancer study, and we have a greater spread of age in the current study, with some stroke cases occurring at 30 years of age. The average age at occurrence in this cohort was just \(60\) years, which is relatively young in the context of stroke.

Both the current study and the Malmo Diet Cancer study are based on post hoc analyses. This is a limitation, and the possibility of chance findings can never be completely ruled out. Further, there might, as always, be unknown confounders. However, of note is that adjustments for available confounders do not weaken our results. The associations were confirmed by use of receiver operating characteristic–curve analysis. Future studies on various populations are warranted to reliably and consistently estimate risk associations.

Persons who developed stroke and incidentally had atrial fibrillation were included among stroke cases because the study design does not make distinctions between subtypes of stroke. This is partly because it is sometimes hard to identify cardioembolic stroke cases and partly because the majority (85%) of ischemic strokes are attributable to atherosclerosis anyway. The number of possible atrial fibrillation–related strokes were most likely not very common because our stroke cases are comparably young, and atrial fibrillation is rather uncommon in this age group.

In a previous side project, we determined IgM anti-PC levels in a population living a traditional life unaffected by a Western lifestyle from Kitava, New Guinea. In this population, CVD in the form of stroke and myocardial infarction is not known, and this is not likely to be related to a short life span because the expected lifetime for adults is relatively long. We compared these persons with Swedish controls, for whom IgM anti-PC was significantly lower. We hypothesized that differences in diet factors and in exposure to

### Table 4. Association Between Low Anti-PC Levels and Risk for Stroke in Men

<table>
<thead>
<tr>
<th>Anti-PC Levels (Men and Women)</th>
<th>Crude</th>
<th>Adjusted for Confounders*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percentiles</td>
<td>Units/mL</td>
<td>No. of Cases=227</td>
</tr>
<tr>
<td>50</td>
<td>51.910</td>
<td>66</td>
</tr>
<tr>
<td>40</td>
<td>44.278</td>
<td>52</td>
</tr>
<tr>
<td>30</td>
<td>35.989</td>
<td>43</td>
</tr>
<tr>
<td>20</td>
<td>29.402</td>
<td>25</td>
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<tr>
<td>10</td>
<td>22.273</td>
<td>13</td>
</tr>
<tr>
<td>4</td>
<td>17</td>
<td>7</td>
</tr>
</tbody>
</table>

*Smoking habits, body mass index (\(>28.14\)), diabetes, hypercholesterolemia (serum cholesterol \(\geq 5.0\) mmol/L), and hypertension (\(\geq 140/90\) mm Hg systolic/diastolic).

OR indicates odds ratio; AUC, area under curve; ROC, receiver operating characteristic curve.

### Table 5. Association Between Low Anti-PC Levels and Risk for Stroke in Women

<table>
<thead>
<tr>
<th>Anti-PC Levels (Men and Women)</th>
<th>Crude</th>
<th>Adjusted for Confounders*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percentiles</td>
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<td>50</td>
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</tr>
<tr>
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<td>11</td>
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<tr>
<td>4</td>
<td>17</td>
<td>5</td>
</tr>
</tbody>
</table>

*Smoking habits, body mass index (\(>28.14\)), diabetes, hypercholesterolemia (serum cholesterol \(\geq 5.0\) mmol/L), and hypertension (\(\geq 140/90\) mm Hg systolic/diastolic).

OR indicates odds ratio; AUC, area under curve; ROC, receiver operating characteristic curve.
microorganisms including nematodes and parasites may contribute.30

Women have significantly higher anti-PC levels than men in all our cohorts for which this comparison has been made,18,19,30 suggesting that higher anti-PC levels may be one factor that could contribute to the later onset of atherosclerosis and CVD in women compared with men.

In general, anti-PCs have been studied in the context of infectious disease because PC is also an epitope found on a multitude of pathogens.31 It was reported recently that among monoclonal antibodies against oxidized low-density lipoprotein from the apolipoprotein E–knockout mouse, one of these, E06, was identical to the anti-PC antibody T15,32 renowned for protecting mice against otherwise fatal Strep-
tococcus pneumonia infections.33 Subsequent in vitro experiments have shown that such anti-PC from mice inhibits foam cell formation by inhibiting macrophage uptake of oxidized low-density lipoprotein.32 Both passive and active immunization trials in murine atherosclerosis models have shown encouraging results, decreasing atherosclerosis develop-
ment.34,35 It could thus be postulated that the defense against PC is a branch of innate immunity that deals with the clearance of both endogenous oxidation-specific epitopes and foreign pathogen-related antigens. Thus, it is possible that low anti-PC levels could implicate an immunodeficient state.

The mechanisms by which anti-PC could protect against stroke could be related to the anti-inflammatory properties of anti-PC, inhibiting the inflammatory effects of PC-containing inflammatory phospholipids including platelet-activating factor, which we reported recently.36 Because proinflammatory cytokines in plaques are abundantly produced by immune competent cells, especially close to sites prone to plaque rupture,2 anti-PC could in principle protect from CVD and stroke by this anti-inflammatory effect.

In conclusion, we report here that low levels of IgM anti-PC predicted an increased risk for stroke in a population-based study from Northern Sweden, with a median age at inclusion of 54 years and median time to first event 5.5 years. In subgroup analyses, this association was only present among women. The findings presented here may potentially open up for novel immunotherapy regimens against athero-
sclerosis and stroke or other forms of CVD either through passive transfer of anti-PC antibodies or active immunization using PC as an antigen. Additional large-scale studies are needed to determine the exact role of anti-PC in women and men and in relation to age.

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Disclosures
J.F. and U.dF. are named as coinventors on pending patent applica-
tions owned by Athera (cofounded by J.F. and U.dF.) relating to anti-PC.

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