Autoantibodies Against Oxidatively Modified LDL Do Not Constitute a Risk Factor for Stroke
A Nested Case-Control Study

Ejaz Ahmed, MD; Jasmina Trifunovic, MSc; Birgitta Stegmayr, MD, PhD; Göran Hallmans, MD, PhD; Ann Kari Lefvert, MD, PhD

Background and Purpose—Autoantibodies against oxidatively modified LDL have been shown to be associated with atherosclerosis. Their possible pathogenic role is not yet fully understood, and earlier published data are inconsistent. In this prospective study, we have investigated the association of these antibodies with future stroke.

Methods—A prospective case-control study in which 44 725 men and women from the World Health Organization Multinational Monitoring of Trends and Determinants in Cardiovascular Disease (MONICA) project and the Västerbotten Intervention Program (VIP) were enrolled and followed from January 1, 1985, to August 31, 1996. One hundred nineteen cases of stroke (male 75, female 44) were noted and compared with 233 age- and sex-matched controls from the same population. Antibodies against oxidatively modified LDL (copper-oxidized LDL and malonaldehyde [MDA]-LDL) were analyzed by ELISA.

Results—There was no difference in the levels or in the prevalence of IgG, IgA, and IgM autoantibodies against copper-oxidized LDL or MDA-LDL between patients and controls. Risk ratios for these antibodies, when adjusted for diabetes mellitus, hypertension, and smoking habits, did not confer a risk of stroke. Serum triglycerides (1.7 versus 1.4 mmol/L, \( P = 0.01 \)), fasting blood sugar, and systolic and diastolic blood pressures were significantly higher in the patient group than in the control group, as was the prevalence of hypertension (51.8% versus 27.4%, \( P < 0.0001 \)) and diabetes mellitus (9.6% versus 0.8%, \( P < 0.001 \)).

Conclusions—Autoantibodies against oxidatively modified LDL do not constitute a risk factor for stroke in an adult population. (Stroke. 1999;30:2541-2546.)

Key Words: autoantibodies n cerebrovascular disorders n epidemiology n lipoproteins, LDL n oxidants

The principal cause of cerebrovascular diseases is atherosclerosis. The local lesion in atherosclerosis is characterized by accumulation of lipid and lipoprotein particles in the subendothelial space and by macrophage and T-lymphocyte infiltration and proliferation of smooth muscle and connective tissue. Oxidative modification of LDL (oxLDL) is essential for macrophage uptake and cellular accumulation of cholesterol, and oxLDL has been suggested to play an important role in atherogenesis. OxLDL is immunogenic and induces the formation of autoantibodies. Atherosclerotic lesions contain immunoglobulins that specifically recognize oxLDL, and autoantibodies against oxLDL may be measured in human plasma. Several studies suggest that higher autoantibody levels against oxLDL may be predictive of atherosclerosis and related diseases, such as coronary heart disease, carotid atherosclerosis, peripheral vascular disease, and diabetes mellitus. The antibodies also occur in hypertension and hyperlipidemia. However, there are also studies showing no association between autoantibodies against oxLDL and the extent of atherosclerosis. Seemingly contradictory results were obtained in experimental animals, in which immunization with LDL and oxLDL had a protective effect against the development of atherosclerotic lesions. Thus, the pathophysiological role of autoantibodies against oxLDL in atherogenesis is not yet fully understood. There is no previous study of the predictive value of the autoantibodies against oxLDL and malonaldehyde-modified LDL (MDA-LDL) for stroke. We report here a prospective study of autoantibodies against native LDL (nLDL), oxLDL, and MDA-LDL in an adult population who developed stroke after an average of 34.1 months and compared them with age- and sex-matched controls.

Subjects and Methods

Study Populations
The 2 northernmost counties in Sweden, with a total population of 510 000, constitute (since 1985) one of 39 collaborating centers in the World Health Organization (WHO) Monitoring of Trends and
Determinants in Cardiovascular Disease (MONICA) study. Population-based surveys were performed in 1986, 1990, and 1994. At each survey, a total of 2000 individuals were invited. In total, 4725 men and women aged 25 to 64 years participated in the MONICA surveys. A community intervention program, the Västerbotten Intervention Program (VIP), for cardiovascular disease and diabetes prevention was performed in 1 of the 2 counties. This program was begun in 1985, and men and women were asked to participate in a health survey (the same design as the MONICA population surveys) the year they reached the age of 30, 40, 50, or 60. In total, ~40,000 men and women participated in this survey between January 1, 1985, and August 31, 1996. Participants in both the MONICA and the VIP surveys were requested to donate a blood sample (minimum 4-hour fasting time) to be stored at the Northern Sweden Medical Bank for future research.

Case Finding
The case finding of stroke was based mainly on 3 sources: discharge records from hospitals, reports from general practitioners, and death certificates. Clinical information on all subjects in the 25- to 74-year range with International Classification of Diseases (8th revision, used from 1985 to 1986 [ICD-8]; 9th revision, used from 1987 to present [ICD-9]) codes 430 to 438 was screened and validated for acute stroke events that met the definition. All cases with acute stroke (group aged 25 to 74 years) and acute myocardial infarction (group aged 25 to 64 years) from the MONICA area have, since 1985, been included in the Northern Sweden MONICA registries. Stroke cases were registered in a standardized way in accordance with the MONICA manual and were defined by the WHO criteria as “rapidly developing clinical signs of focal (or global) disturbance of cerebral function lasting more than 24 hours (unless interrupted by surgery or death) with no apparent cause other than a vascular origin.” This definition excludes transient ischemic attacks. Global clinical signs were accepted only in patients with deep coma or subarachnoid hemorrhage. Subdural hemorrhage, traumatic intracerebral hemorrhage, and lesions caused by brain tumor were also excluded. In this longitudinal project, special emphasis was placed on using uniform case ascertainment and diagnostic criteria throughout the study. Case finding was therefore based on clinical presentation, and cases detected by brain imaging but not exhibiting any acute symptoms of stroke were excluded. Information was abstracted from medical records, and the codes were the same throughout the study. Every case was strictly validated according to MONICA criteria before registration.

The subtypes of acute stroke, according to the MONICA manual, were based on the following examinations (ICD-9 codes are in parentheses): intracerebral hemorrhage (431), positive finding on CAT scan or necropsy; brain infarction (434), no signs of hemorrhage on CAT scan or at necropsy; and unspecified stroke (436), not investigated by CAT scan or necropsy. In this study, 3 of 119 patients had a diagnosis of unspecified stroke, and they were categorized as having “ischemic stroke” in the analysis.

Each case was also described as “definite stroke,” “unclassifiable,” or “not stroke” on the basis of available background information. “Unclassifiable” was mainly used in fatal cases for which acute cerebrovascular disease was given as cause of death in the death certificate but for which clinical information was too limited to classify the event as “definite stroke.” The proportion of “unclassifiable” cases was 7.5% of fatal strokes and 1.4% of all strokes, with no important changes over time. Fatal cases were subjects who died within 28 days from the onset of the stroke.

A nested case-referent design was used when the incident cases were defined first-ever stroke events, as classified according to MONICA criteria and identified during the period ranging from January 1, 1985, to August 31, 1996. We identified 166 individuals who, after participation in either the MONICA or VIP health surveys, suffered from a first-ever ischemic or hemorrhagic stroke before the age of 74. For this study, 119 cases (98 ischemic and 21 hemorrhagic; 75 men and 44 women) remained after the exclusion of individuals with a previous myocardial infarction (n=15) or stroke (n=9) or cancer diagnosis according to the Regional Cancer registry (n=13); individuals were also excluded if the amount of blood in the sample taken was inadequate for analysis (n=10).

Potential referents for each case were randomly selected among participants in the MONICA or the VIP survey. They were matched for sex, age (±2 years), type of survey (MONICA or VIP), date (±1 year) of health survey, and geographical region. Individuals were excluded if they had died or had moved away from the Northern MONICA region before August 31, 1996. Referents were also excluded if they were known from the Northern Sweden MONICA incidence registry to have had acute myocardial infarction or stroke before the health survey. An additional questionnaire was sent to all referents to further ensure the absence of stroke and/or acute myocardial infarction in their histories. Finally, 2 referents for each case were selected. However, 5 patients had only 1 corresponding matched control.

Smokers were defined as those who reported smoking daily. Exsmokers and “occasional smokers” were classified as nonsmokers. Smokeless tobacco (oral snuff) users were defined as snuffers if they used snuff daily.

Blood pressure was recorded after 5 minutes of rest. Hypertension was defined as systolic blood pressure ≥160 mm Hg and/or diastolic blood pressure ≥95 and/or antihypertensive medication. Total cholesterol was measured by use of a bench-top analyzer (Reflotron, Boehringer Mannheim GmbH Diagnostica) or by an enzymatic method (Boehringer Mannheim GmbH Diagnostica).

Specimen Collection
Blood was drawn by clean venipuncture. Specimens were centrifuged, and plasma was immediately frozen and stored at ~80°C until the assay was performed. The study was approved by the Research Ethics Committee of Umeå University.

Lipoprotein Isolation and MDA Modification of LDL
Lipoprotein isolation and MDA modification of LDL were performed according to published methods.

Oxidation of LDL by Copper
Oxidation of LDL by copper was performed as described earlier.

Determination of Antibodies Against oxLDL, MDA-LDL, and nLDL
Antibodies of IgG, IgM, and IgA isotypes were determined by ELISA according to a published method. Patient and corresponding age- and sex-matched control assays were run at the same time with known positive and negative serum samples. Levels of antibodies were expressed in optical density (OD) units, and the prevalence of antibodies was expressed as values above mean ± 2 SD of the control population. The assay had been further validated by using F(ab')2 fragments from purified IgG fractions with high concentration of specific antibodies.

Statistical Analysis
The SAS Statistical Software Package was used for data analysis (SAS Institute Inc, 1995). Conditional logistic regression analyses were performed to determine the most powerful predicting factors with respect to the outcome measure. For each predicting factor (explanatory variable), the 95% confidence limits for the conditional risk ratio (e^b) were calculated. The conditional logistic regression is a stratified analysis, in which each matched set is a stratum. The Fisher exact test was used for the comparison of categorical variables, and a test was used for the comparison of continuous variables.

Results
Table 1 summarizes the clinical data, prevalence of autoantibodies, and stroke risk factors in patients and controls. Serum triglycerides, fasting blood sugar, and both systolic and diastolic blood pressures (not shown in table) were significantly higher in the patient group than in the control
TABLE 1. Clinical Characteristics and Prevalence of Autoantibodies Against oxLDL, MDA-LDL, and nLDL in Stroke Patients and Controls

<table>
<thead>
<tr>
<th></th>
<th>Patients (N=119)</th>
<th>Controls (N=233)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age, y</td>
<td>55.1±7.7</td>
<td>55±7.7</td>
<td>0.851</td>
</tr>
<tr>
<td>Sex, % female</td>
<td>36.9 (44/119)</td>
<td>36.4 (85/233)</td>
<td>1</td>
</tr>
<tr>
<td>Autoantibodies</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-oxLDL, IgG, %</td>
<td>7.5 (9/119)</td>
<td>5.1 (12/233)</td>
<td>0.354</td>
</tr>
<tr>
<td>Anti-oxLDL, IgM, %</td>
<td>8.4 (10/119)</td>
<td>4.2 (10/233)</td>
<td>0.143</td>
</tr>
<tr>
<td>Anti-oxLDL, IgA, %</td>
<td>4.2 (5/119)</td>
<td>3.4 (8/233)</td>
<td>0.768</td>
</tr>
<tr>
<td>Anti–MDA-LDL, IgG, %</td>
<td>3.3 (4/119)</td>
<td>3.8 (9/233)</td>
<td>1</td>
</tr>
<tr>
<td>Anti–MDA-LDL, IgM, %</td>
<td>3.3 (4/119)</td>
<td>4.2 (10/233)</td>
<td>0.779</td>
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<td>Anti–MDA-LDL, IgA, %</td>
<td>2.5 (3/119)</td>
<td>3.4 (8/233)</td>
<td>0.756</td>
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</tbody>
</table>

Risk factors

<table>
<thead>
<tr>
<th></th>
<th>Patients (N=119)</th>
<th>Controls (N=233)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypertension, %</td>
<td>51.8 (56/108)</td>
<td>27.4 (58/211)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Diabetes mellitus, %</td>
<td>9.6 (11/114)</td>
<td>0.8 (2/226)</td>
<td>0.0002</td>
</tr>
<tr>
<td>Cigarette smoking, %</td>
<td>25.8 (30/116)</td>
<td>17.4 (40/229)</td>
<td>0.088</td>
</tr>
<tr>
<td>Smokeless tobacco, %</td>
<td>7.2 (8/110)</td>
<td>12.8 (28/218)</td>
<td>0.139</td>
</tr>
</tbody>
</table>

Other characteristics

<table>
<thead>
<tr>
<th></th>
<th>Patients (N=119)</th>
<th>Controls (N=233)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body mass index</td>
<td>26.9±4.2</td>
<td>26±3.8</td>
<td>0.088</td>
</tr>
<tr>
<td>Serum cholesterol, mmol/L</td>
<td>6.4±1.3</td>
<td>6.2±1.3</td>
<td>0.348</td>
</tr>
<tr>
<td>HDL, mmol/L</td>
<td>1.2±0.3</td>
<td>1.3±0.3</td>
<td>0.585</td>
</tr>
<tr>
<td>Serum triglycerides, mmol/L</td>
<td>1.7±1</td>
<td>1.4±0.6</td>
<td>0.017</td>
</tr>
</tbody>
</table>

Values are mean±SD or percentages (n/N).
§Information is not known for all patients or controls.

There were no differences in body mass index, total serum cholesterol, and HDL.

The prevalence of hypertension (51.8% versus 27.4%, \( P<0.0001 \)) and diabetes mellitus (9.6% versus 0.8%, \( P<0.001 \)) was significantly higher in patients than in controls, but smoking habits and use of smokeless tobacco did not differ between the groups. The prevalence of autoantibodies against oxLDL, MDA-LDL, and nLDL did not differ between the patients and the controls.

The Figure shows the autoantibody levels against oxLDL and MDA-LDL. There was no significant difference in the autoantibody levels against oxLDL and MDA-LDL between patients with cerebral hemorrhage and patients with cerebral infarction or between all patients and controls.

Table 2 presents the data obtained by using the calculation method proposed by Salonen et al.\(^{10}\) There was no significant difference between the stroke patients and the controls for the ratio of anti-oxLDL to nLDL or for the ratio of anti–MDA-LDL to nLDL.

Table 3 summarizes the risk of stroke related to autoantibody levels when adjusted for diabetes mellitus, hypertension, smoking habits, and use of smokeless tobacco. Autoantibodies against oxLDL and MDA-LDL did not confer a risk of stroke. However, diabetes and hypertension conferred a higher risk of stroke.

Female patients had higher levels of autoantibodies against MDA-LDL of the IgM type than did the male patients (\( P<0.01 \)). However, there was no significant difference between the female patients and the corresponding female controls in the autoantibody levels. Female patients had a higher levels of serum triglycerides than did the female controls (\( P<0.05 \)).

There was no significant difference between the patients with cerebral hemorrhage and the patients with cerebral infarction in the levels of autoantibodies against oxLDL or MDA-LDL or in the ratio of oxLDL to nLDL or the ratio of MDA-LDL to nLDL. There were no differences in body mass index, total serum cholesterol, HDL, and serum triglycerides between the 2 groups.

Discussion

We found no association between stroke and autoantibodies against oxidatively modified lipoproteins in the present prospective study.

In the present study, both oxLDL and MDA-LDL were used as antigens. oxLDL contains various epitopes required for the oxidation process, and MDA-LDL contains MDA-lysine adducts, which represent one class of oxidation-derived antigen epitopes in vivo. In oxLDL, the density of various oxidation-derived epitopes is likely to be lower than that found in MDA-LDL. However, copper-oxLDL represents a more physiological product of LDL. An advantage of using MDA-LDL as an antigen is that antibody levels are generally higher, as shown in a previous study\(^{17}\) and also reported in the present study.

In the present study, we measured autoantibody concentrations in 2 different ways: (1) the level of autoantibodies against oxLDL, MDA-LDL, and nLDL was expressed as OD values,\(^{16}\) and (2) the calculation method proposed by Salonen et al,\(^{10}\) in which autoantibodies are expressed as a ratio (binding to oxLDL and MDA-LDL divided by binding to native LDL, all expressed as OD units) was used. Because both ways of expressing the data generate the same result, it is justifiable to conclude that our results do not indicate any biased method of calculation.

The results of the present study are consistent with reports from several other studies, in which no differences in the levels of autoantibodies against oxLDL were found in patients with non–insulin-dependent diabetes mellitus, coronary heart disease, and stroke,\(^{20–22,29}\) but our results are inconsistent with other studies, in which higher autoantibody levels were found in carotid atherosclerosis, coronary atherosclerosis, essential hypertension, and myocardial infarction.\(^{10,12,18,30}\) Coronary heart disease is the most reliable indicator and a classic example of atherosclerosis, and almost all patients with myocardial infarction have coronary atherosclerosis, whereas cerebrovascular disease (stroke) is a less reliable criterion for the presence of atherosclerosis because it includes both cerebral hemorrhage and cerebral thrombosis. Cerebral hemorrhage is often the result of vascular defects due to hypertension and diabetes. In most studies, elevated levels of autoantibodies against oxLDL are reported mainly in subjects with coronary heart disease, and these studies are predominantly retrospective. Therefore, we cannot rule out the possibility that coronary heart disease may in fact promote the development of these autoantibodies.

In a 10-year follow-up study of patients with non–insulin-dependent diabetes mellitus, Uusitupa et al\(^{20}\) found that...
autoantibodies against oxLDL do not predict cardiovascular mortality or morbidity and that they are not associated with the intimal-medial thickness of the common carotid artery and carotid bifurcation. In a recent study by Cherubini et al., it was found that older patients with stroke in fact have lower autoantibody levels against oxLDL and that this is probably due to binding of these autoantibodies to free radical–modified molecules. Free radicals are believed to play an important role in the pathogenesis of ischemic brain injury.

The present study supports the strength of an association between stroke and traditional risk factors such as hypertension and diabetes mellitus. Smoking can increase the oxidation of LDL, but in the present study, there was no significant difference in smoking between stroke patients and controls. One explanation for not finding a relation between autoantibodies and stroke could be that different persons may have different populations of antibodies. Dietary or other changes in lifestyle may affect LDL oxidation in vivo and

Box plots showing the levels of IgG, IgM, and IgA autoantibodies against oxLDL (A) and MDA-LDL (B) in patients with cerebral hemorrhage (P1) and cerebral infarction (P2) and in controls (C). PS indicates median value of known positive samples. The levels are expressed as OD values. The lower, middle, and upper horizontal lines of the boxes represent 25th, 50th, and 75th percentiles, respectively; the vertical lines extend from the 10th to the 90th percentile.
Autoantibodies in Patients With Stroke

|TABLE 3. Levels of Autoantibodies Against oxLDL and MDA-LDL in Stroke Patients (Risk Ratio Adjusted for Diabetes Mellitus, Hypertension, Cigarette Smoking, and Smokeless Tobacco) |

<table>
<thead>
<tr>
<th>Autoantibodies against oxLDL</th>
<th>Risk Ratio</th>
<th>95% CI Lower</th>
<th>95% CI Upper</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diabetes</td>
<td>17.5</td>
<td>2.097</td>
<td>145.481</td>
</tr>
<tr>
<td>Hypertension</td>
<td>3.3</td>
<td>1.751</td>
<td>6.239</td>
</tr>
<tr>
<td>Smoking</td>
<td>1.7</td>
<td>0.826</td>
<td>3.196</td>
</tr>
<tr>
<td>Smokeless tobacco</td>
<td>0.6</td>
<td>0.194</td>
<td>1.252</td>
</tr>
<tr>
<td>oxLDL IgG</td>
<td>1.1</td>
<td>0.700</td>
<td>1.869</td>
</tr>
<tr>
<td>oxLDL IgM</td>
<td>1.1</td>
<td>0.742</td>
<td>1.766</td>
</tr>
<tr>
<td>oxLDL IgA</td>
<td>1.2</td>
<td>0.704</td>
<td>1.958</td>
</tr>
</tbody>
</table>

Values are mean±SD. Anti-oxLDL IgG, IgM, and IgA ratios were calculated as the ratio between anti-oxLDL and anti-nLDL autoantibodies. Anti-MDA-LDL IgG, IgM, and IgA ratios were calculated as the ratio between anti-MDA-LDL and anti-nLDL autoantibodies as proposed by Salonen et al.10

Autoantibodies against MDA-LDL

<table>
<thead>
<tr>
<th>Autoantibodies against MDA-LDL</th>
<th>Risk Ratio</th>
<th>95% CI Lower</th>
<th>95% CI Upper</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diabetes</td>
<td>18.2</td>
<td>2.149</td>
<td>154.909</td>
</tr>
<tr>
<td>Hypertension</td>
<td>3.2</td>
<td>1.673</td>
<td>6.148</td>
</tr>
<tr>
<td>Smoking</td>
<td>1.7</td>
<td>0.813</td>
<td>3.168</td>
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<tr>
<td>Smokeless tobacco</td>
<td>0.6</td>
<td>0.203</td>
<td>1.619</td>
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<tr>
<td>MDA-LDL IgG</td>
<td>1.5</td>
<td>0.742</td>
<td>2.986</td>
</tr>
<tr>
<td>MDA-LDL IgM</td>
<td>1.1</td>
<td>0.622</td>
<td>2.072</td>
</tr>
<tr>
<td>MDA-LDL IgA</td>
<td>1.2</td>
<td>0.593</td>
<td>2.319</td>
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</table>

References


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