High Proinsulin Levels Precede First-Ever Stroke in a Nondiabetic Population

Bernt Lindahl, MD, PhD; Bo Dinesen, MSc; Mats Eliasson, MD, PhD; Michael Røder, MD; Göran Hallmans, MD, PhD; Birgitta Stegmayr, PhD

Background and Purpose—Diabetic subjects have a 3- to 6-fold increased risk for stroke compared with nondiabetic subjects, and hyperinsulinemia shows strong and consistent associations with a cluster of cardiovascular risk factors. Methods separating proinsulin from (true) insulin have demonstrated proinsulin to be more strongly associated with cardiovascular disease than insulin. The present study evaluates the associations between first-ever stroke, proinsulin, and insulin.

Methods—In this incident case-referent study of a nondiabetic population, 94 cases of first-ever stroke (59 men and 35 women) were individually age- and sex-matched to 178 referents. Blood sampling was collected before the stroke event. Proinsulin and insulin were measured with highly sensitive 2-site sandwich enzyme-linked immunosorbent assays.

Results—In the study population, high proinsulin concentration more than tripled the risk for first-ever stroke after adjustments for total cholesterol, systolic blood pressure, smoking, body mass index, and insulin, with an odds ratio of 3.4 (95% CI, 1.4 to 8.4). In women the risk was even more pronounced, with an odds ratio of 13.7 (95% CI, 1.3 to 146). Synergy was found between proinsulin and systolic blood pressure. In women, synergy was also found between proinsulin and diastolic blood pressure as well as between insulin and both blood pressures.

Conclusions—High levels of proinsulin may predict later occurrence of first-ever stroke in a nondiabetic population. (Stroke. 2000;31:2936-2941.)

Key Words: insulin ■ proinsulin ■ stroke

The underlying cause of stroke in most cases is an atherosclerotic process in the blood vessels, and ischemic stroke accounts for approximately 80% of all stroke events.1 Next to age, hypertension is the most important risk factor for both hemorrhagic and ischemic strokes.2,3 Other important risk factors are smoking, diabetes, and hypercholesterolemia, but none of these are as important as hypertension. Men and women with diabetes have an excessive risk for cerebral infarction, while the risk for hemorrhage does not seem to be elevated.4 In the Framingham Study, 45- to 74-year-old diabetic men and women were found to have 2.5 to 3.5 times higher risk for cerebral infarction than nondiabetic individuals.5 The Northern Sweden MONICA (Monitoring of Trends and Determinants in Cardiovascular Disease) study found a 3-4-fold higher risk for stroke in diabetic compared with nondiabetic men and nearly 6-fold higher risk for diabetic versus nondiabetic women.6 Recently, 2 prospective studies confirmed diabetes to be a strong risk factor for stroke. Furthermore, after exclusion of subjects with diabetes, both studies showed elevated fasting insulin levels to be associated with increased risk of future stroke.7,8

Ample evidence has been presented on the association between the insulin level and a cluster of cardiovascular risk factors. The risk factors that constitute the metabolic syndrome are obesity (especially abdominal), dyslipidemia with high triglyceride and low HDL cholesterol levels, hypertension, and low fibrinolytic activity. Often the syndrome also includes impaired glucose tolerance.9-11

The introduction of specific immunoradiometric12 or enzyme-linked immunosorbent assays (ELISA),13,14 which measure immunoreactivity from proinsulin and split proinsulin separately from true insulin, made it possible to independently examine the effect of proinsulin and true insulin on cardiovascular risk factors and cardiovascular disease. It has already been shown, in both diabetics and nondiabetics, that proinsulin and split proinsulin have a stronger association than insulin to dyslipidemia (high triglycerides and low HDL cholesterol), hypertension, and impaired glucose tolerance.15 It has also been shown in vitro that proinsulin, at least as strongly as insulin and independently of insulin, increases the level of plasminogen activator inhibitor type 1 (PAI-1) activity and thereby lowers fibrinolytic activity.16,17 Furthermore, the association between proinsulin and intima-media wall thickness in the common carotid artery was stronger than the corresponding association between insulin and intima-media wall thickness.18
The aim of the present study was to evaluate proinsulin and insulin as risk markers for first-ever stroke in a non-diabetic population.

Subjects and Methods

Study Population

Since 1985, in Västerbotten County in northern Sweden, there has been an ongoing community intervention program for cardiovascular disease and diabetes, the Västerbotten Intervention Program (VIP). As a part of this program, all men and women were invited to a health survey at the age of 30, 40, 50, and 60 years. At the same time, the 2 northernmost counties in Sweden (Västerbotten and Norrbotten) joined the World Health Organization (WHO) MONICA study. Within this project, population-based health surveys were performed in 1986, 1990, and 1994. In total, 4725 men and women in the group aged 25 to 64 years participated. In both the VIP and the MONICA surveys, participants were requested to donate a blood sample to be stored at the Northern Sweden Medical Research Bank for future research purposes. In the present study a nested case-referent design has been used, in which incident cases of first-ever stroke were defined by the Northern Sweden MONICA incidence registry. The inclusion criteria for the cases were a first-ever stroke classified according to the MONICA criteria and identified during the period of January 1, 1985, through August 31, 1996. An additional criterion was the participation in the VIP or the MONICA survey and the presence of a blood sample in the Northern Sweden Medical Research Bank before the stroke event. One hundred twelve survey and the presence of a blood sample to be stored at the Northern Sweden Medical Research Bank for future research purposes. In the present study a nested case-referent design has been used, in which incident cases of first-ever stroke were defined by the Northern Sweden MONICA incidence registry. The inclusion criteria for the cases were a first-ever stroke classified according to the MONICA criteria and identified during the period of January 1, 1985, through August 31, 1996. An additional criterion was the participation in the VIP or the MONICA survey and the presence of a blood sample in the Northern Sweden Medical Research Bank before the stroke event. One hundred twelve cases remained after the exclusion of individual cases with a previous acute myocardial infarction (n = 15), stroke (n = 9), or cancer diagnosis (n = 13) and of cases in which the blood sample taken was inadequate for analysis (n = 17).

Two referent subjects for each case were randomly selected among participants in the VIP or MONICA surveys. They were matched for sex, age (± 2 years) and date (± 1 year) of health survey, and geographic region. Individuals were excluded if they had died or had moved away from the region on or before August 31, 1996. The referent subjects were also excluded if they had an acute myocardial infarction, stroke, or cancer before the health survey. A questionnaire was sent to all referents to further ensure absence of stroke and/or acute myocardial infarction in their history.

For the present study all individuals (cases and referents) with known (self-reported) diabetes (n = 11) or unknown diabetes (n = 18) were excluded. Unknown diabetes was defined as having a fasting plasma glucose at the health survey of ≥7 mmol/L or a 2-hour plasma glucose during an oral glucose tolerance test (OGTT) in the diabetic range, i.e., ≥12.2 mmol/L in capillary plasma. An OGTT was performed in >90% of the participants. Twenty-nine diabetic subjects (18 cases and 11 referents) were excluded along with the individually matched referents (n = 36) to the stroke cases with diabetes. Hence, 94 cases (59 men and 35 women) and 178 referents (113 men and 65 women) remained and formed the basis of the present study.

Methods

At the health survey, blood pressure was measured after the subject had rested for 5 minutes in the recumbent position. Body weight was measured with the subject in light indoor clothing and recorded to the nearest kilogram. Height was measured to the nearest centimeter and without shoes. Body mass index was calculated as weight (kilograms) divided by height (meters) squared. Smokers were defined as those reporting daily smoking of cigarettes, cigarillos, cigars, or a pipe. Ex-smokers and occasional smokers were classified as non-smokers. During the first years of the VIP health surveys, the minimum fasting period before blood sampling was determined to be 4 hours. Since most of the health surveys were done in the morning, a majority of the participants had an overnight fast. In 1992 and subsequent years the requested minimum fasting period was changed to 8 hours. In a majority of the subjects (n = 252), an abbreviated OGGT was performed according to the WHO standard, with a 75-g anhydrous glucose load and with measurement of plasma glucose after 2 hours. Venous blood was taken in heparin tubes to obtain plasma for analyses of proinsulin and insulin. The plasma samples were frozen within 1 hour at −20°C and later during the day or within a week stored at −80°C. Fresh serum was used for measurement of total cholesterol.

Laboratory Procedures

Proinsulin was measured with the use of a highly sensitive 2-site sandwich ELISA. The assay is based on 2 monoclonal antibodies, a mouse anti-human C-peptide antibody (PEP-001) and a mouse anti-human insulin antibody (HUI-001). The detection limit in human serum is 0.25 pmol/L. There was no cross-reaction with human insulin and human C-peptide. However, the 4 major proinsulin conversion intermediates reacted in various proportions ranging from 65% to 99%. Insulin was measured in a similar manner with the use of another sensitive 2-site sandwich ELISA. The assay is based on 1 monoclonal antibody with its epitope near the C-terminal end of the B-chain (OXI-005) and 1 monoclonal antibody with its epitope centered around the A-chain loop (HUI-018). The detection limit is 5.0 pmol/L. The specificity of the assay excludes intact, split(32–33)–, and des(31,32)-proinsulin. There was some cross-reaction with split(65–66)-proinsulin (30%) and des(64,65)-proinsulin (63%). Fasting plasma glucose, 2-hour plasma glucose during an OGTT, and total serum cholesterol concentrations were measured with Reflotron bench-top analyzers (Boehringer Mannheim GmbH) at the time of the health survey.

The study was approved by the Research Ethics Committee at Umeå University, and the data handling procedures were approved by the National Computer Data Inspection Board.

Statistical Analysis

The Statistical Analysis System (SAS) version 6.12 and EGRET software version 1.01.10 were used. Our main variables (proinsulin and insulin) had a skewed distribution. An approximate normal distribution was achieved after logarithmic transformation, and all statistical analyses assuming normal distribution were performed on the transformed values. The sample was divided into tertiles (proinsulin, insulin, systolic and diastolic blood pressure) defined by the distribution of these variables among the referent subjects. This procedure was done separately for the whole study population as well as for men and women. Univariate conditional logistic regression analyses were performed to estimate odds ratios (ORs) and 95% CIs of having a first-ever stroke for different levels of proinsulin, insulin, and blood pressure. Multiple conditional logistic regression analyses were conducted on the association between first-ever stroke and proinsulin or insulin. Adjustments were made for cardiovascular risk factors. The presence of synergy was tested by dichotomizing and combining proinsulin or insulin and blood pressure. A synergy index (SI) was calculated (SI = RR Ab−1/RR aB−1 + RR aB−1) as proposed by Rothman. Synergy was indicated when SI exceeded the value of 1.

Five subjects (4 men and 1 woman) had missing values in the smoking variable, and an additional 6 subjects (2 men and 4 women) had missing values in the blood pressure variables and were excluded from the conditional logistic regression analyses. Four subjects (1 man and 3 women) had missing values in the insulin variable. Nine subjects (all men) had a missing value in body mass index. These subjects remained in the regression analyses, and their missing value in BMI was replaced by the mean of the male referents.

Results

Ninety-four cases (59 men and 35 women) with first-ever stroke together with 178 referents (113 men and 65 women) were included in the study. Basal characteristics are presented in Table 1. In the whole study population, the systolic and...
diastolic blood pressures along with the proinsulin level were significantly elevated in the cases compared with the referents. In men cases had higher blood pressure and higher body mass index than referents, whereas in women cases had higher proinsulin and insulin levels.

Significant correlations between proinsulin, insulin, and other study variables were found (Table 2). In both men and women, proinsulin and insulin correlated with body mass index. Proinsulin also correlated with diastolic blood pressure. In women, proinsulin and insulin correlated with systolic blood pressure. In general, the correlations in women were stronger than in men.

Using proinsulin as a continuous variable in the conditional logistic regression analyses showed in the whole study population a 4.8% increase in risk of first-ever stroke for every picomole per liter of elevation in proinsulin. In women the corresponding risk increase was 16%. The same kind of calculation using insulin was only marginally significant, with a risk increase for the whole population of 0.1% and in women of 0.6% for every picomole per liter of elevation in insulin.

To further estimate the relative risk of first-ever stroke in subjects with high compared with low levels of proinsulin, insulin, or blood pressure, the whole study population was divided into tertiles on the basis of the distribution of the risk variable in the referents. By conditional logistic regression analyses, crude ORs and their 95% CIs were calculated (Table 3). Values in the top tertile of proinsulin compared with the bottom tertile indicated a more than doubled risk of future stroke, with an OR of 2.6 (95% CI, 1.3 to 5.3). For insulin the OR was 2.0 (95% CI, 1.0 to 4.0). The corresponding estimate for top versus bottom tertile for systolic blood pressure was more than 5 times higher, with an OR of 5.6 (95% CI, 2.2 to 13.8), and the estimate for diastolic blood pressure was more than 10 times higher, with an OR of 10.2 (95% CI, 3.4 to 31.0). In women but not in men, an excess risk for stroke due to elevated proinsulin or insulin levels was seen (Table 3).

Multiple regression analyses were used to estimate the association of proinsulin or insulin with first-ever stroke after adjustment for potential confounders, such as smoking, total cholesterol, body mass index, and systolic or diastolic blood pressure. Proinsulin continued to explain excess risk of stroke in the whole study population as well as in women when systolic blood pressure was included in the regression model. However, proinsulin disappeared as an explanatory variable for stroke when systolic blood pressure was exchanged for diastolic blood pressure. Adding insulin (as a continuous variable) to the model resulted in minor changes in the ORs of proinsulin and in slightly larger CIs. High levels of proinsulin still indicated an excess risk for stroke in women when systolic blood pressure was exchanged for diastolic blood pressure, with an OR of 2.6 (95% CI, 1.3 to 5.3). For insulin the OR was 2.0 (95% CI, 1.0 to 4.0). The corresponding estimate for top versus bottom tertile for diastolic blood pressure was more than 5 times higher, with an OR of 5.6 (95% CI, 2.2 to 13.8), and the estimate for diastolic blood pressure was more than 10 times higher, with an OR of 10.2 (95% CI, 3.4 to 31.0). In women but not in men, an excess risk for stroke due to elevated proinsulin or insulin levels was seen (Table 3).

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variables were created. The first of them combined proinsulin and systolic blood pressure. Before this, proinsulin was dichotomized into the top tertile versus the middle and bottom tertiles, and systolic blood pressure was divided into $\geq 140$ versus $<140$ mm Hg. Of the 4 possible contingencies, low proinsulin combined with low blood pressure was used as reference. ORs and 95% CIs were calculated by conditional logistic regression analyses. SI was manually calculated. In the whole study population, as well as in men and women separately, synergy was found between proinsulin and systolic blood pressure, with SIs of 1.6 to 1.7. A corresponding interaction variable was created between proinsulin and diastolic blood pressure with a cutoff of 85 mm Hg. In women, synergy was found with a SI of 1.7. In a similar way, positive interaction between insulin and systolic or diastolic blood pressure was tested. Synergy was found in the whole study group between insulin and systolic blood pressure (SI 1.4). There was also an interaction in women between insulin and both systolic and diastolic blood pressures, with SIs of 2.1 and 1.3, respectively.

**Discussion**

The present study indicates that high proinsulin levels may increase the risk for first-ever stroke. The association was seen in women as well as in the study population as a whole. The relation was clearly weaker and nonsignificant in men. The mean level of proinsulin among referents in this study of 7.0 pmol/L was similar to that of a healthy population from the same area of Sweden (6.9 pmol/L), suggesting that the referent group of this study on average has a normal level of proinsulin. Women having proinsulin levels $\geq 8.3$ pmol/L (top tertile) compared with women with levels $\leq 5.1$ pmol/L (bottom tertile) were associated with a 4-fold increase in risk. The corresponding risk in the whole study group was more than doubled (OR 2.6). The risk factor status of proinsulin in regard to first-ever stroke was still present after controlling for the traditional cardiovascular risk factors of total cholesterol, smoking, systolic blood pressure, and body mass index. However, it disappeared when diastolic blood pressure replaced systolic blood pressure in the model. Additional control in the model for insulin did not significantly change the result. Women with an insulin level $\geq 44$ pmol/L (top tertile) compared with women with an insulin level $<28$ pmol/L (bottom tertile) showed a substantially increased risk for first-ever stroke after adjustment for potential confounders, with an OR of 2.7 (95% CI, 1.7 to 4.3). However, the excess risk disappeared when proinsulin was added to the model. There was also indication of synergy between proinsulin and systolic blood pressure, with a SI of 1.6 to 1.7. The interaction effect was seen in the whole study population as well as in men and women. Synergy between insulin and systolic blood pressure was also found in the whole study population as well as in women.

### Table 3. Crude ORs and 95% CIs for First-Ever Stroke in Different Tertiles of Proinsulin, Insulin, and Systolic and Diastolic Blood Pressure

<table>
<thead>
<tr>
<th></th>
<th>Proinsulin</th>
<th>Insulin</th>
<th>Systolic Blood Pressure</th>
<th>Diastolic Blood Pressure</th>
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<tr>
<td>All (n=261)</td>
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<td>1.0</td>
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<tr>
<td>Middle</td>
<td>1.2 (0.6–2.5)</td>
<td>1.1 (0.6–2.3)</td>
<td>1.9 (0.8–4.6)</td>
<td>7.5 (2.6–22.2)</td>
</tr>
<tr>
<td>Top</td>
<td>2.6 (1.3–5.3)</td>
<td>2.0 (1.0–4.0)</td>
<td>5.6 (2.2–13.8)</td>
<td>10.2 (3.4–31.0)</td>
</tr>
<tr>
<td>Men (n=166)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bottom</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Middle</td>
<td>1.0 (0.4–2.4)</td>
<td>0.9 (0.4–2.0)</td>
<td>2.2 (0.7–7.2)</td>
<td>18.4 (2.4–141)</td>
</tr>
<tr>
<td>Top</td>
<td>2.0 (0.9–4.7)</td>
<td>1.2 (0.5–2.8)</td>
<td>5.1 (1.6–16.5)</td>
<td>25.0 (3.2–197)</td>
</tr>
<tr>
<td>Women (n=95)</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bottom</td>
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<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
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<tr>
<td>Middle</td>
<td>1.8 (0.4–8.1)</td>
<td>5.9 (0.7–51.8)</td>
<td>1.0 (0.2–5.4)</td>
<td>6.2 (0.7–52.4)</td>
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<td>9.7 (1.2–77.8)</td>
<td>6.3 (1.5–28.6)</td>
<td>19.9 (2.2–180)</td>
</tr>
</tbody>
</table>

**Table 4.** ORs and 95% CIs for First-Ever Stroke in Different Tertiles of Proinsulin Before and After Adjustments for Potential Confounders

<table>
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<tr>
<th></th>
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<th>Adjusted (1) OR</th>
<th>Adjusted (2) OR</th>
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<td>All (n=257)</td>
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<td></td>
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<tr>
<td>$\leq 5.3$</td>
<td>18</td>
<td>56</td>
<td>1.0</td>
<td>1.0</td>
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<tr>
<td>5.4–7.7</td>
<td>21</td>
<td>56</td>
<td>1.3 (0.6–3.0)</td>
<td>1.3 (0.5–3.0)</td>
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<tr>
<td>$\geq 7.8$</td>
<td>46</td>
<td>60</td>
<td>3.4 (1.4–8.4)</td>
<td>2.6 (1.0–6.7)</td>
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<td>Men (n=165)</td>
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</tr>
<tr>
<td>$\leq 5.3$</td>
<td>14</td>
<td>34</td>
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<td>1.0</td>
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<tr>
<td>5.4–7.6</td>
<td>15</td>
<td>39</td>
<td>0.9 (0.3–2.3)</td>
<td>0.8 (0.3–2.4)</td>
</tr>
<tr>
<td>$\geq 7.7$</td>
<td>26</td>
<td>37</td>
<td>1.6 (0.5–4.9)</td>
<td>2.0 (0.6–7.2)</td>
</tr>
<tr>
<td>Women (n=92)</td>
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</tr>
<tr>
<td>$\leq 5.1$</td>
<td>4</td>
<td>21</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>5.2–8.2</td>
<td>7</td>
<td>18</td>
<td>3.9 (0.5–32.8)</td>
<td>2.1 (0.3–12.9)</td>
</tr>
<tr>
<td>$\geq 8.3$</td>
<td>19</td>
<td>23</td>
<td>13.7 (1.3–146)</td>
<td>4.3 (0.6–31.6)</td>
</tr>
</tbody>
</table>

Adjusted (1) OR adjusted for total cholesterol, systolic blood pressure, smoking, body mass index, and insulin; adjusted (2), same as (1) but systolic replaced by diastolic blood pressure. Proinsulin is measured in picomoles per liter.
The mechanisms by which proinsulin and possibly insulin may contribute to the development of first-ever stroke are incompletely understood. One leading hypothesis focuses on the role of hemostasis in general and especially on the associations with disturbed fibrinolysis. Proinsulin has been shown, at least as strongly as insulin and independently of insulin, to increase the level of PAI-1 activity, thereby lowering fibrinolytic activity. At the same time, we must realize that the low concentration of proinsulin in the circulation among nondiabetic individuals would contradict the hypothesis of a direct biological effect on stroke by proinsulin. Another possible, and perhaps more plausible, explanation for the relation between proinsulin and first-ever stroke is that the level of proinsulin acts indirectly as a sensitive marker of an underlying metabolic disturbance reflecting the actual demand on the β cells.

Our study supports earlier findings of a reduced sex-related difference in cardiovascular risk when diabetic or metabolically disturbed (high proinsulin levels) men and women are compared with metabolically normal men and women. In the 20-year follow-up of the Framingham Study, the incidence of every measured cardiovascular event was higher in normal men than in normal women. However, in the study diabetic women had a higher incidence of stroke than diabetic men.

It is important to emphasize the incident case-referent design of this study, implying that the exposure factors were measured before disease was developed. This minimizes recall and selection biases and excludes the possibility that high proinsulin levels were an effect of a previous stroke. The identification and definition of cases in a case-referent design are crucial, and it should be stressed that all stroke events in this study were strictly classified according to the MONICA criteria by the Northern Sweden MONICA stroke registry. Extensive quality assessments of the registry have been performed.

It is not easy to compare the impact of different cardiovascular risk factors in the etiology of a first-ever stroke. In a multifactorial disease, several different causative mechanisms are present. In this mosaic of more or less related risk factors, some of them will be in the same causal path, while others will be joined in another causal path, and often we do not know the exact relations between our measured risk factor variables and to which causal path they belong. Proinsulin and insulin are secreted together from the β cell and probably exert their biological effects in the body independently of each other. This does not exclude the possibility of coinciding effects later in the causal path of stroke (e.g., PAI-1 activity). Therefore, when we examined the association between proinsulin and stroke, it seemed appropriate to include insulin in the regression model, and vice versa. However, the addition of 2 highly correlated variables, such as proinsulin and insulin, in the same model may create a situation of near collinearity. In our study the addition of insulin to the model only resulted in minor changes in the ORs of proinsulin on stroke, indicating that the multiple regression model chosen from a statistical point of view seemed robust enough for this procedure. Body mass index and proinsulin were also highly correlated, and it is certainly biologically plausible that these 2 variables act in the same causal path in the etiology of a first-ever stroke. The use of multiple regression analyses emphasizes the need of careful interpretation of the results as well as of which variables should be included in the regression models.

In conclusion, high concentrations of proinsulin in this incident case-referent study predicted a >2-fold increase, and in women a >4-fold increase, of first-ever stroke in nondiabetic individuals.

Acknowledgments

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