Proinsulin Concentration Is an Independent Predictor of All-Cause and Cardiovascular Mortality

An 11-year follow-up of the Hoorn Study

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Objective — High proinsulin concentration may be a better predictor for cardiovascular disease (CVD) mortality than insulin concentration. Previous observations may have been confounded by glucose tolerance status or lack of precision because of high intra-individual variability. We investigated the longitudinal relation of means of duplicate measurements of insulin and proinsulin with all-cause and CVD mortality in a population-based cohort taking glucose tolerance status into account.

Research Design and Methods — Fasting and post–75-g glucose-load (2-h) glucose, insulin, and proinsulin values were determined in duplicate on separate days in 277 participants with normal glucose metabolism, 208 participants with impaired glucose metabolism, and 119 newly detected patients with type 2 diabetes of the Hoorn Study. Insulin resistance and β-cell function were estimated by homeostasis model assessment (HOMA-IR and HOMA-B, respectively), and the fasting proinsulin-to-insulin ratio was calculated. Subjects were followed with respect to mortality until January 2003.

Results — Fasting proinsulin levels were significantly associated with all-cause and CVD mortality. The hazard ratios (HRs) per increase in interquartile range adjusted for age and sex were 1.21 (95% CI 1.04–1.42) for all-cause mortality and 1.33 (1.06–1.66) for CVD mortality. Adjustment for glucose tolerance status and HOMA-IR did not substantially change the associations.

Conclusions — Fasting proinsulin was associated with all-cause and CVD mortality, independent of glucose tolerance status and insulin resistance and largely independent of other CVD risk factors. Proinsulin might play a role in the relationship between insulin resistance and CVD.

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glucose tolerance status in the relation between insulin or proinsulin and mortality.

All analyses were done in a population-based cohort that was stratified for glucose tolerance status.

**RESEARCH DESIGN AND METHODS** — The Hoorn Study is a population-based cohort study on type 2 diabetes in the general Dutch population. The study population and research design have been described in detail previously (15). In summary, in 1989, 3,553 men and women, aged 50–75 years, were randomly selected from the population register of the middle-sized Dutch town of Hoorn. Of the 2,540 subjects (71.5%) who agreed to participate, 56 non-Caucasians were excluded. Therefore, the study cohort consisted of 2,484 men and women. All subjects gave written informed consent. The Ethics Committee of the VU University Medical Center approved the study. After exclusion of patients with known diabetes (n = 90), as defined by use of insulin or blood glucose–lowering agents, or a prescribed diet for diabetes, a subgroup of 1,109 participants from the initial study cohort, stratified by age, sex, and 2-h postload plasma glucose values, was invited for a second oral glucose tolerance test. For reasons of efficiency, insulin and proinsulin were measured in duplicate (~2 weeks apart) in a subsample of 630 participants of the stratified sample of 1,109 participants, including participants with impaired glucose tolerance and all participants with newly detected diabetes (15). Subjects with values below the lower limit of sensitivity of the insulin or proinsulin assay were excluded. For the present analyses, subjects were included if either insulin or proinsulin values were available in duplicate (n = 605). Of these 605 subjects, one subject was lost to follow-up. Thus, the present study population comprised 604 subjects. A total of 602 participants had duplicate insulin values, and proinsulin values were available for 511 participants.

Anthropometric measurements were obtained from all participants. Weight was measured while participants were wearing light clothes only. Participants were not wearing shoes while height was measured. BMI was calculated as weight (in kilograms) divided by the square of height (in meters). Waist circumference (centimeters) was measured following a standardized procedure (15).

Systolic and diastolic blood pressure were determined at the right upper-arm, after 5 min of rest in seated participants, with a random-zero sphygmomanometer (Hawksley-Gelman, Lancing, U.K.). The average of duplicate measurements was used.

All laboratory analyses were performed at the VU University Medical Center in Amsterdam. Fasting plasma glucose concentration and 2-h postload plasma glucose concentration were determined by the glucose dehydrogenase method (Merck, Darmstadt, Germany). Immuno-specific insulin was measured in serum by a double-antibody radio immunoassay (lot SP21; Linco Research, St. Louis, MO) in which proinsulin and 33,33 split pro-insulin cross-reacts by <0.2%. Proinsulin was measured by a double-antibody radioimmunnoassay (Lilly Laboratory for Clinical Research, Indianapolis, IN) in which 31,32 proinsulin cross-reacts by 63% (18). Triglycerides, total cholesterol, and HDL cholesterol measured on the first visit were determined by enzymatic techniques (Boehringer-Mannheim, Mannheim, Germany).

**Mortality follow-up**

There is a continuous follow-up to register mortality from the participants of the Hoorn Study, with the municipal register of the city of Hoorn providing information about the vital status of the participants. Information of causes of death was obtained from medical records of general practitioners and from the local hospital. Causes of death were coded according to the ICD-9 (19). CVD mortality was defined as ICD-9 codes 390–459 (diseases of the circulatory system) or 798 (sudden death, cause unknown) because, generally, sudden death is of cardiovascular origin (20). The mortality follow-up was completed by January 2003.

**Statistical methods**

Glucose tolerance status (based on fasting and postload glucose values) and fasting and postload insulin and proinsulin values were based on the mean of two measurements. If only one value was determined, values were based on one measurement. This was done for 1 subject for fasting plasma glucose, 16 subjects for postload plasma glucose, 16 subjects for fasting insulin, and 61 subjects for fasting proinsulin.

The mean fasting and postload glucose values of duplicate oral glucose tolerance tests were used to define the glucose tolerance status of the participants (21). Insulin resistance was estimated by HOMA-IR, calculated as fasting insulin (in microunits per milliliter) × fasting glucose (in millimoles per liter)/22.5. β-Cell function was estimated by HOMA-B, calculated as fasting insulin × 20/fasting glucose (in millimoles per liter) – 3.5 (16).

Due to skewed distributions, baseline characteristics are shown as medians with interquartile ranges. Differences in baseline measurements for groups of glucose tolerance status were examined using linear regression for continuous data and logistic regression for dichotomous data, adjusted for age. Correlations among insulin, proinsulin, HOMA-IR, and HOMA-B were calculated by Spearman correlation coefficients because of skewed distribution of insulin and proinsulin.

Hazard ratios (HRs) and 95% CIs were obtained from multivariate Cox proportional hazards models. HRs are calculated for one increase in interquartile range value, which is calculated for each variable. All models were adjusted for age and sex. We tested for possible interaction between insulin, proinsulin, HOMA-IR, and glucose tolerance status by adding two product terms to the survival models insulin × impaired glucose metabolism and insulin × diabetes. We also tested for possible interaction among insulin, proinsulin, HOMA-IR, and sex because this was found in some studies (2). In the second model, we additionally adjusted for glucose tolerance status as a potential confounding factor. Third, we also adjusted for insulin resistance, calculated as HOMA-IR, to investigate the effect of insulin and proinsulin levels on (CVD) mortality independent of HOMA-IR. To find other potential confounding or mediating factors, we added fasting and postload glucose, BMI, triglycerides, HDL cholesterol, total cholesterol, hypertension, and current smoking one by one to the third model. We adjusted our model for the variables that affected the estimated association. A P value <0.05 was considered statistically significant. Testing for interaction, we considered a P value <0.10 statistically significant. Statistical analyses were performed with the SPSS software package for Windows (version 10.1.4).
RESULTS — The characteristics of the study cohort, stratified for glucose tolerance status, are shown in Table 1. Of the 604 participants, 156 died, of whom 59 died from CVD or sudden death during the 11 years of follow-up. There was an increasing risk of all-cause mortality over the glucose tolerance groups (P = 0.04). For CVD mortality, increase in risk over glucose tolerance groups was not statistically significant (P = 0.10). All other variables, except sex and total cholesterol, showed statistically significant differences (P < 0.05) across glucose tolerance groups.

Spearman correlation coefficients among insulin, proinsulin, HOMA-IR, and HOMA-B are shown in Table 2. All shown variables were statistically significant and correlated with each other (P < 0.01), except fasting proinsulin with HOMA-B and proinsulin-to-insulin ratio with postload insulin and with HOMA-IR. Proinsulin-to-insulin ratio was statistically significant but inversely related to fasting insulin and HOMA-B.

**Table 1—Baseline characteristics in median values (interquartile range) and mortality in categories of glucose tolerance status (n = 604)**

<table>
<thead>
<tr>
<th></th>
<th>NGM*</th>
<th>IGM</th>
<th>New diabetes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>n</strong></td>
<td>277</td>
<td>208</td>
<td>119</td>
</tr>
<tr>
<td><strong>Baseline</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>63.8 (11.7)</td>
<td>64.7 (12.6)</td>
<td>66.2 (10.4)</td>
</tr>
<tr>
<td>Sex (% male)</td>
<td>48.4</td>
<td>48.6</td>
<td>47.9</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>25.6 (4.2)</td>
<td>27.4 (4.7)</td>
<td>28.1 (5.1)</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>89.5 (13.2)</td>
<td>95.0 (15.0)</td>
<td>99.5 (15.8)</td>
</tr>
<tr>
<td>Fasting glucose (mmol/l)†</td>
<td>5.30 (0.55)</td>
<td>6.05 (0.79)</td>
<td>7.40 (1.75)</td>
</tr>
<tr>
<td>2-h postload glucose (mmol/l)†</td>
<td>5.75 (2.23)</td>
<td>8.50 (1.54)</td>
<td>12.65 (4.95)</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>1.30 (0.80)</td>
<td>1.70 (1.00)</td>
<td>2.00 (1.50)</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/l)</td>
<td>1.35 (0.49)</td>
<td>1.17 (0.47)</td>
<td>1.10 (0.36)</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>6.60 (1.50)</td>
<td>6.80 (1.50)</td>
<td>6.50 (1.70)</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)†</td>
<td>80.0 (13.0)</td>
<td>83.0 (11.5)</td>
<td>84.3 (11.5)</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)†</td>
<td>130.5 (22.0)</td>
<td>140 (27.4)</td>
<td>144 (20.8)</td>
</tr>
<tr>
<td>Hypertension (%)‡</td>
<td>27.5</td>
<td>48.6</td>
<td>55.5</td>
</tr>
<tr>
<td>Current smoker (%)</td>
<td>31.4</td>
<td>21.6</td>
<td>21.0</td>
</tr>
<tr>
<td>Fasting-specific insulin (pmol/l)†</td>
<td>72.1 (36.8)</td>
<td>88.6 (53.9)</td>
<td>117.2 (70.4)</td>
</tr>
<tr>
<td>Fasting proinsulin (pmol/l)†</td>
<td>9.5 (7.9)</td>
<td>14.2 (12.9)</td>
<td>21.7 (20.0)</td>
</tr>
<tr>
<td>Proinsulin-to-insulin ratio†</td>
<td>0.12 (0.11)</td>
<td>0.14 (0.13)</td>
<td>0.16 (0.14)</td>
</tr>
<tr>
<td>Postload-specific insulin (pmol/l)†</td>
<td>310.5 (260.4)</td>
<td>532.6 (542.7)</td>
<td>600.5 (567.3)</td>
</tr>
<tr>
<td>Postload proinsulin (pmol/l)†</td>
<td>49.8 (49.2)</td>
<td>74.9 (63.4)</td>
<td>93.4 (67.2)</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>2.80 (1.55)</td>
<td>4.01 (2.47)</td>
<td>6.61 (4.53)</td>
</tr>
<tr>
<td>HOMA-B</td>
<td>137.4 (75.5)</td>
<td>123.2 (85.8)</td>
<td>104.4 (81.3)</td>
</tr>
<tr>
<td><strong>11-year follow-up</strong></td>
<td></td>
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</tr>
<tr>
<td>All-cause death [n (%)]</td>
<td>59 (21.3)</td>
<td>55 (26.4)</td>
<td>42 (35.3)</td>
</tr>
<tr>
<td>CVD mortality or sudden death [n (%)]</td>
<td>20 (7.2)</td>
<td>22 (10.6)</td>
<td>17 (14.3)</td>
</tr>
</tbody>
</table>

Values within parentheses represent interquartile ranges unless otherwise indicated. Linear regression for continuous variables: P < 0.05, except for total cholesterol (P = 0.98). Logistic regression for percentages: P < 0.05, except for sex (P = 0.69) and CVD mortality (P = 0.10). *Glucose tolerance status based on the mean fasting and 2-h postload glucose levels of two oral glucose tolerance tests; †based on the mean of two measurements; ‡hypertension (diastolic blood pressure ≥95 mmHg or systolic blood pressure ≥160 mmHg or medication). NGM, normal glucose metabolism (fasting glucose ≤6.1 mmol/l and 2-h glucose ≤7.8 mmol/l); IGM, impaired glucose metabolism (fasting glucose ≥6.1 and <7.0 mmol/l or 2-h glucose ≥7.8 and ≤11.1 mmol/l); diabetes, fasting glucose ≥7.0 mmol/l or 2-h glucose ≥11.1 mmol/l; HOMA-IR, fasting insulin (in microunits per milliliter) × fasting glucose (in millimoles per liter)/22.5; HOMA-B, fasting insulin (in microunits per milliliter) × 20/fasting glucose (in millimoles per liter) − 3.5.

**Table 2—Spearman correlation coefficients for insulin and proinsulin, HOMA-IR, and HOMA-B**

<table>
<thead>
<tr>
<th></th>
<th>Fasting-specific insulin</th>
<th>Fasting proinsulin</th>
<th>Proinsulin-to-insulin ratio</th>
<th>Postload-specific insulin</th>
<th>Postload proinsulin</th>
<th>HOMA-IR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fasting proinsulin</td>
<td>0.47*</td>
<td>0.78*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proinsulin-to-insulin ratio</td>
<td>0.14*</td>
<td>0.43*</td>
<td>0.41*</td>
<td>0.74*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Postload-specific insulin</td>
<td>0.67*</td>
<td>0.09*</td>
<td>−0.34*</td>
<td>0.44*</td>
<td>0.25*</td>
<td>0.38*</td>
</tr>
<tr>
<td>Postload proinsulin</td>
<td>0.51*</td>
<td>0.45*</td>
<td></td>
<td>0.63*</td>
<td>0.52*</td>
<td></td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>0.95*</td>
<td>0.53*</td>
<td>−0.04</td>
<td>0.63*</td>
<td>0.52*</td>
<td></td>
</tr>
<tr>
<td>HOMA-B</td>
<td>0.63*</td>
<td>0.09*</td>
<td>−0.34*</td>
<td>0.44*</td>
<td>0.25*</td>
<td>0.38*</td>
</tr>
</tbody>
</table>

*Correlation is statistically significant (P < 0.01). HOMA-IR, fasting insulin (in microunits per milliliter) × fasting glucose (in millimoles per liter)/22.5; HOMA-B, fasting insulin (in microunits per milliliter) × 20/fasting glucose (in millimoles per liter) − 3.5.
In Table 3, HRs per increase in interquartile range was associated with a 21% higher all-cause mortality and a 33% higher CVD mortality. The association of proinsulin with mortality risk appeared stronger than that of insulin with mortality risk. This has also been observed in another longitudinal study (9) and in cross-sectional studies (22,23). Moreover, in a model that included both proinsulin and insulin or HOMA-IR, proinsulin remained significantly associated with (CVD) mortality, whereas insulin or HOMA-IR did not remain statistically significant. Only one previous study considered insulin as a covariate (11). In that study, proinsulin remained an independent risk factor for stroke, and the excess risk of insulin disappeared when proinsulin was added to the model.

High proinsulin levels can be a result of insulin resistance or β-cell failure, and both may contribute to the association between proinsulin and CVD. Proinsulin has been shown to be strongly associated
Proinsulin and (cardiovascular) mortality

with insulin resistance in a recent study (24), which may at least partly explain the association with CVD and mortality (25,26). A strong correlation between proinsulin and HOMA-IR can cause multicollinearity when both variables are included in the same model. In the present study, the Spearman correlation coefficient between proinsulin and HOMA-IR was 0.53, substantially lower than the 0.90 cutoff value, which is often used as a rule of thumb to define multicollinearity. We found that the association between proinsulin and (CVD) mortality was independent of insulin resistance. In contrast, the association between HOMA-IR and CVD mortality disappeared following adjustment for proinsulin. Therefore, high proinsulin levels might be an intermediate factor in the association between insulin resistance and CVD risk, in which high proinsulin levels are more closely related to CVD than insulin resistance.

High proinsulin levels may also be attributed to β-cell dysfunction or an increased demand on the β-cells (14). In the current study, disproportionately elevated proinsulin-to-insulin ratios or HOMA-B (16) were not associated with an increased risk of CVD mortality, suggesting that proinsulin per se may contribute to the atherogenic or thrombogenic process. Indeed, a clinical study in which human proinsulin was administered for at least 1 year was stopped when six patients treated with human proinsulin had adverse cardiovascular events (13), whereas subjects treated with insulin had no adverse events.

In the IRAS (Insulin Resistance Atherosclerosis Study), plasminogen activator inhibitor-1 (PAI-1) was suggested to play a role in the association between proinsulin and CVD (22). Increased PAI-1 activity promotes both fibrosis and thrombosis and is associated with CVD (27). It was already shown that PAI-1 activity increased after insulin or proinsulin was administered in vitro (28) and in vivo (29). Unfortunately, in the Hoorn Study, data on PAI-1 are not available. PAI-1 levels are elevated in subjects with insulin resistance, impaired glucose metabolism, and the metabolic syndrome (27). Therefore, the metabolic syndrome, which is defined by high triglycerides, impaired glucose metabolism, hypertension, abdominal obesity, and/or low HDL (30), is another potential confounding factor in the relation between proinsulin and CVD risk. However, in our data, triglyceride concentration, glucose tolerance status, and hypertension explained only a small part of the association between proinsulin and CVD risk.

Hyperinsulinemia has been shown to predict all-cause (1) and CVD mortality (2,4,5,8) in the general population. We did find an association between insulin and CVD mortality, similar to the findings in the meta-analysis of the DECODE (Diabetes Epidemiology: Collaborative Analysis of Diagnostic Criteria in Europe) Insulin Study Group. However, in the present study, the association between insulin and CVD mortality was not significant, probably because of the relatively small number of case subjects.

In a number of studies, the association between insulin and coronary heart disease was J-shaped (3) or U-shaped (31). In the present subpopulation of the Hoorn Study, the number of cases was too small to explore this association. When we analyzed the entire Hoorn Study population, baseline insulin levels were available for 1,806 participants with normal glucose metabolism. Indeed, the HRs for fasting-specific insulin of the lowest quintile against the middle three quintiles, adjusted for age and sex, were 1.10 (95% CI 0.81–1.50) for all-cause mortality and 1.07 (0.65–1.75) for CVD mortality (unpublished data). For the present analysis, we did not consider this a relevant deviation of a linear association.

In some studies, postload insulin levels, 2 h after a 75-g glucose load, have been associated with coronary artery disease (7,8). In the present study, postload insulin levels were not significantly associated with mortality, and the association was weaker than between fasting insulin and mortality. These results are consistent with findings from a recent meta-analysis (2). The weaker association between postload insulin and mortality was explained by the larger biological variation of postload insulin levels. In our study, biological variation was reduced by measuring insulin levels in duplicate, but this could still play a role in attenuating the association between postload insulin and mortality.

We did not use gold standard techniques for measuring insulin sensitivity (i.e., the hyperinsulinemic-euglycemic clamp) and for β-cell function (i.e., the hyperglycemic clamp) (32). Instead, HOMA-IR was used as an estimate of insulin resistance (16). In previous studies, HOMA-IR was found to be a predictor of CVD in diabetic patients (23,33). However, HOMA-IR cannot be used to estimate insulin resistance in diabetic patients (34). Therefore, patients with known diabetes were excluded from the present study. Our observation that insulin levels and HOMA-IR were the strongest predictors of all-cause and CVD mortality in subjects with a normal glucose tolerance status might be explained by the fact that insulin and HOMA-IR are less accurate indexes of insulin resistance in patients with impaired glucose metabolism or newly detected type 2 diabetes.

A strength of our study is that levels of insulin, proinsulin, and glucose were measured twice to improve the precision. We included participants with newly diagnosed diabetes and with impaired glucose metabolism to enlarge the range of distribution of insulin, proinsulin, HOMA-IR, and HOMA-B. Also, these subjects are especially important to include because of their enhanced CVD risk.

In our study, proinsulin concentration was associated with both all-cause and CVD mortality, independent of glucose tolerance status and insulin resistance. In addition, we showed that the association between HOMA-IR and all-cause mortality disappeared when proinsulin was entered into the model. High proinsulin levels might therefore play a role in the atherothrombotic process, possibly as an intermediate in the association between insulin resistance and CVD risk.

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References


