High Serum Levels of Advanced Glycation End Products
Predict Increased Coronary Heart Disease Mortality in
Nondiabetic Women but not in Nondiabetic Men
A Population-Based 18-Year Follow-Up Study

Bente K. Kilhovd, Auni Juutilainen, Seppo Lehto, Tapani Rönnemaa, Peter A. Torjesen,
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Background—Advanced glycation end products (AGEs), modification products of glycation or glycoxidation of proteins and lipids, have been linked to premature atherosclerosis in patients with diabetes as well as in nondiabetic subjects.

Methods and Results—Serum levels of AGEs were measured with an immunoassay in samples obtained at baseline examination of a random sample of 1141 nondiabetic individuals (535 men and 606 women), aged 45 to 64 years, living in Kuopio, East Finland, or Turku, West Finland in 1982 to 1984. After 18 years of follow-up, all-cause mortality, cardiovascular disease (CVD), and coronary heart disease (CHD) mortality were registered on the basis of copies of death certificates. Multivariate Cox regression model showed a significant association of serum AGEs with all-cause (P = 0.012), CVD (P = 0.018), and CHD (P = 0.008) mortality in women but not in men. Fasting serum AGEs in the highest quartile were an independent risk factor for all-cause (hazards ratio [HR], 1.90; 95% CI, 1.16 to 3.11; P = 0.011) and CHD (HR, 6.51; 95% CI, 1.78 to 23.79; P = 0.005) mortality in women, even after the adjustment for confounding factors, including highly sensitive C-reactive protein.

Conclusions—The present study is the first to show that serum levels of AGEs can predict total, CVD, and CHD mortality in nondiabetic women.

Key Words: advanced glycation end products ▪ coronary heart disease ▪ cardiovascular disease ▪ mortality

Advanced glycation end products (AGEs), the short- and long-term modification products of glycation or glycoxidation of proteins and lipids,1,2 have been linked to premature atherosclerosis in diabetic patients3 as well as in nondiabetic subjects.4 AGEs are a heterogeneous group of compounds that have multiple biological effects, some of which are mediated by interacting with receptors, including the receptor for AGE (RAGE), on endothelial cells, smooth muscle cells, and macrophages.5–7 Furthermore, AGEs have been found in atherosclerotic plaques.8 AGEs may contribute to development of atherosclerosis by activating the transcription factor nuclear factor κB (NF-κB) through RAGE binding, resulting in induction of cellular adhesion molecule expression and cytokine activation,9,10 or through glycoxidation of lipoproteins and increased foam cell formation.11,12 AGEs might also quench NO and mediate impaired endothelial function.13 Increased AGE modification of long-lived proteins such as collagen increases cross-linking and stiffening of arteries.14 Experimental studies in animals and in humans have shown that a cross-link breaker treatment results in greater vascular compliance.15,16 Recently, elevated levels of circulating AGEs were reported in nondiabetic patients with coronary heart disease (CHD) that correlated significantly with the number of vessels with stenosis.17

Because no study has been undertaken to examine whether there is any association of serum levels of AGEs with the subsequent development of cardiovascular disease (CVD) in a large nondiabetic population, the present study was aimed to investigate whether increased serum levels of AGEs predict total mortality, CVD mortality, or CHD mortality in Finnish nondiabetic subjects.

Materials and Methods

Baseline Study

A random sample of nondiabetic subjects born and currently living in Kuopio, East Finland, or in Turku, West Finland, was taken from the population register including all individuals aged 45 to 64 years in 1982 through 1984. Of the 827 individuals in eastern Finland and 863 in western Finland originally eligible for the study, a total of 638 men and 735 women were examined: 313 men and 336 women from Eastern Finland (participation rate 79%) and 325 men and 399 women from Western Finland (participation rate 88%).

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women from West Finland (participation rate 85%). All subjects with a previous history of diabetes or newly diagnosed diabetes according to the World Health Organization (WHO) criteria at the baseline study were excluded from the present analyses. Clinical characteristics and background variables did not differ between participants and nonparticipants.

The study program for subjects was performed during an outpatient visit at the clinical research unit of the University of Kuopio or the rehabilitation research center of the Social Insurance Institution in Turku. These methods have been described previously. The visit included an interview on the history of chest pain suggestive of CHD, smoking, alcohol intake, physical activity, and drug use. All medical records of subjects who reported on the interview that they had been admitted to hospital for chest pain symptoms were reviewed. Review of the medical records was performed by M.L. and T.R. in Turku and K.P. in Kuopio after careful standardization of the methods between the reviewers. The WHO criteria for verified definite or possible myocardial infarction (MI), based on chest pain symptoms, ECG changes, and enzyme determinations, were used to define previous MI.

Smoking status was based on an interview. In all statistical analyses, subjects were classified as nonsmokers or current smokers. Blood pressure was measured in the sitting position after a 5-minute rest with a mercury sphygmomanometer and read to the nearest 2 mm Hg. Subjects were classified as having hypertension if they were receiving drug treatment for hypertension or if their systolic blood pressure was $\geq$160 mm Hg or diastolic blood pressure $\geq$100 mm Hg.

All laboratory specimens were drawn after a 12-hour fast at 8:00 AM and subsequently stored frozen at $-20^\circ$C until analysis. Serum levels of AGEs were measured with a competitive immunoassay developed in our laboratory. The method has been described previously. Briefly, we used polyclonal anti-AGE antibodies from rabbit immunized with AGE-RRNase. Europium-labeled anti-ribag IgG was used as indicator, and AGE-BSA was used as standard. Triplicates of standard or sample together with a fixed amount of anti-AGE antibody were added to wells coated with AGE-BSA. Plates were incubated while shaking for 2 hours, washed, and then indicated antibodies were added. After another hour of incubation, Europium chelate–delayed fluorescence was measured. One AGE unit was defined as the displacement activity of 1 $\mu$g/ml AGE-BSA standard.

The final serum concentrations of AGE were adjusted for total protein concentration. Interassay coefficient of variation was 15% for the control in the median range of the assay curve and 24% in the lower range. Two batches of antibody obtained from the same animal were used in the measurements. No significant differences between the batches were found. Serum from a total of 1141 persons was available for AGE measurement.

Fasting plasma glucose was determined by the glucose oxidase method (Boehringer Mannheim). Serum lipids and lipoproteins were determined from fresh serum samples. Serum total cholesterol (intra-assay variation 1.6%) and triglycerides (intra-assay variation 2.6%) was assayed by automated enzymatic methods (Boehringer Mannheim). Serum high-density lipoprotein (HDL) cholesterol (intra-assay variation 1.7%) was determined enzymatically after precipitation of low-density lipoprotein (LDL) and very LDL particles with dextran sulfate-MgCl$_2$. LDL cholesterol was calculated using the Friedewald formula. Total protein concentration (inter-assay variation 4.5%) was measured with the Coomassie brilliant blue method (Bio-Rad). Highly sensitive C-reactive protein (hs-CRP) was determined in 468 men and 531 women by latex turbidimetric immunoassay (Wako Chemicals). Analytical detection limit of the assay was 0.06 mg/mL, and the interassay coefficient of variation was 3.33% at the mean level of 1.5 mg/L and 2.65% at the mean level of 2.5 mg/L.

Follow-Up Study

The follow-up period was until January 1, 2001. Information on the vital status of the participants and copies of death certificates of all deceased subjects were obtained from the Cause-of-Death Register (Statistics Finland). In the final classification of causes of death,
higher total cholesterol (6.92 ± 1.27 versus 6.74 ± 1.32 mmol/L; \(P=0.048\)), total triglycerides (1.48 ± 0.74 versus 1.40 ± 0.74 mmol/L; \(P=0.032\)), and lower HDL cholesterol (1.44 ± 0.39 versus 1.50 ± 0.39 mmol/L; \(P=0.023\)) than subjects in other quartiles; whereas no difference in age, BMI, or the prevalence of hypertension was observed. There were no statistically significant correlations between serum AGE level and age, fasting plasma glucose, total cholesterol, HDL cholesterol, total triglycerides, and BMI (range of correlations from 0.015 to 0.081 in men and from 0.022 to 0.079 in women). The lack of significant correlations is not surprising given the fact that absolute levels of cardiovascular risk factors were not substantially different between the highest quartile of AGEs versus the other quartiles. Exclusion of 2 patients from statistical analyses who had serum creatinine >200 μmol/L did not change the results.

Table 2 gives unadjusted and multiple-adjusted hazard ratios (HRs) for serum levels of AGEs as a continuous variable to predict all-cause, non-CVD, CVD, and CHD mortality (Cox regression model). Because AGE levels and gender had a statistically significant interaction in their effects on total mortality (\(P=0.023\)) and CHD mortality (\(P=0.012\)), even after the adjustment for confounding variables, results are presented separately for men and women. Unadjusted serum AGEs were significantly related to total (\(P=0.007\)), CVD (\(P<0.001\)), and CHD (\(P<0.001\)) mortality in women but not in men. Adjusted HRs remained essentially similar.

Total and CHD mortality in men and women according to the gender-specific AGE quartiles (6.0 U/mL, 7.6 U/mL, and 9.9 U/mL in men; 5.6 U/mL, 7.1 U/mL, and 9.0 U/mL in women) is shown in Figure 1. The highest quartile of serum AGEs versus the 3 other quartiles predicted total (\(P=0.032\)) and CHD mortality (\(P=0.001\)) in women but not in men (Figure 2). In women, high AGE levels increased mortality from all causes by 1.6-fold (\(P=0.042\)) and CHD mortality by 4-fold (\(P=0.014\)), independently of confounding factors. AGE levels were not associated with increased mortality from noncardiovascular causes (Table 3). Additional adjustment for hs-CRP increased further the HRs for all-cause mortality.

### Table 2. Unadjusted and Adjusted HRs and their 95% CIs From Cox Proportional Hazards Model for AGEs (per 1-unit increment) in Relation to Total, Non-CVD, CVD, and CHD Mortality During an 18-Year Follow-Up in 535 Nondiabetic Men and 606 Nondiabetic Women (n=1141)

<table>
<thead>
<tr>
<th></th>
<th>Unadjusted</th>
<th>P Value</th>
<th>Adjusted</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR (95% CI)</td>
<td></td>
<td>HR (95% CI)</td>
<td></td>
</tr>
<tr>
<td><strong>Total mortality</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All</td>
<td>1.03 (0.99–1.06)</td>
<td>0.150</td>
<td>1.01 (0.98–1.05)</td>
<td>0.524</td>
</tr>
<tr>
<td>Men</td>
<td>0.98 (0.93–1.02)</td>
<td>0.323</td>
<td>0.98 (0.94–1.03)</td>
<td>0.517</td>
</tr>
<tr>
<td>Women</td>
<td>1.07 (1.02–1.13)</td>
<td>0.007</td>
<td>1.08 (1.02–1.14)</td>
<td>0.012</td>
</tr>
<tr>
<td><strong>Non-CVD mortality</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All</td>
<td>1.01 (0.96–1.06)</td>
<td>0.758</td>
<td>1.00 (0.95–1.05)</td>
<td>0.996</td>
</tr>
<tr>
<td>Men</td>
<td>0.97 (0.90–1.04)</td>
<td>0.367</td>
<td>0.97 (0.90–1.04)</td>
<td>0.392</td>
</tr>
<tr>
<td>Women</td>
<td>1.04 (0.97–1.11)</td>
<td>0.290</td>
<td>1.04 (0.97–1.13)</td>
<td>0.274</td>
</tr>
<tr>
<td><strong>CVD mortality</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All</td>
<td>1.05 (1.00–1.10)</td>
<td>0.069</td>
<td>1.03 (0.98–1.09)</td>
<td>0.286</td>
</tr>
<tr>
<td>Men</td>
<td>0.98 (0.92–1.05)</td>
<td>0.609</td>
<td>1.00 (0.94–1.06)</td>
<td>0.993</td>
</tr>
<tr>
<td>Women</td>
<td>1.13 (1.05–1.20)</td>
<td>&lt;0.001</td>
<td>1.10 (1.02–1.20)</td>
<td>0.018</td>
</tr>
<tr>
<td><strong>CHD mortality</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All</td>
<td>1.06 (1.01–1.12)</td>
<td>0.027</td>
<td>1.05 (0.98–1.11)</td>
<td>0.145</td>
</tr>
<tr>
<td>Men</td>
<td>1.00 (0.93–1.07)</td>
<td>0.891</td>
<td>1.01 (0.94–1.08)</td>
<td>0.825</td>
</tr>
<tr>
<td>Women</td>
<td>1.14 (1.07–1.23)</td>
<td>&lt;0.001</td>
<td>1.13 (1.03–1.23)</td>
<td>0.008</td>
</tr>
</tbody>
</table>

The Cox model was adjusted for age, area of residence, sex (in all), BMI, current smoking, hypertension, total cholesterol, triglycerides, HDL cholesterol, and menopausal status (in women).
mortality up to 1.9-fold ($P=0.011$) and CHD mortality up to 6.5-fold ($P=0.005$).

In Kaplan–Meier survival analysis, total ($P=0.032$) and CHD mortality ($P<0.001$) were significantly higher in subjects in the highest AGE quartile compared with the 3 other quartiles in women (Figure 2). In men, no differences in mortality according to the AGE quartiles were observed.

**Discussion**

The present study is the first to show that increased AGE serum levels, measured with a polyclonal anti-AGE antibody, can predict total, CVD, and CHD mortality, and that serum AGE level in the top quartile ($9.0$ U/mL) is an independent risk factor for total and CHD mortality in nondiabetic women.

AGE-modified proteins are a heterogeneous group of compounds formed through glycation, glycoxidation, or, like N’-(carboxymethyl)lysine (CML), through glycoxidation/oxidation from lipids. The dominant AGE epitope for binding to the RAGE receptor is CML. Through binding to the RAGE receptor, CML may induce NF-κB activation and vascular cell adhesion molecule-1 expression, which might contribute to accelerated atherosclerosis. Circulating AGES may arise from intracellularly formed reactive AGE precursors, and AGES can be formed in the circulation, for example, on lipids. We measured serum AGES with a polyclonal anti-AGE antibody to detect most of the circulating AGES. The polyclonal anti-AGE antibody has been shown previously to recognize CML, as well as a number of as yet unidentified AGE epitopes. Thus, variations in AGE levels could mirror changes in CML levels that might be related to inflammation through NF-κB activation, reflecting some of the burden of inflammation as well as that of glucose-modification of proteins.

Our finding of a significant association of AGE serum levels with CHD and CVD mortality in women might indicate an etiologic role of AGES in atherosclerosis in patients without diabetes. So far, only 1 animal study has demonstrated a causal involvement of AGES in atherosclerosis. In that study, diabetic apolipoprotein E (apoE)–null mice were given normal chow, which increased atherosclerosis, but by adding soluble RAGE, development of atherosclerosis was almost completely inhibited. There was also a nonsignificant trend toward a reduction in atherosclerosis in nondiabetic apoE-null mice given soluble RAGE in addition to normal chow compared with those continuing on normal chow. Recently, treatment with soluble RAGE significantly reduced atherosclerotic lesion area in apoE-null diabetic mice having established atherosclerosis.

Fasting serum AGES in the highest quartile ($9.0$ U/mL) in this population-based study were an independent risk factor for total and CHD mortality in women. High AGE levels might reflect some of the increase in inflammation or

**TABLE 3. High Levels of Serum AGES as a Risk Factor for Total, CHD, and Non-CVD Mortality in 1141 Nondiabetic Subjects During 18-Year Follow-Up**

<table>
<thead>
<tr>
<th>Adjustment</th>
<th>Total Mortality</th>
<th>CHD Mortality</th>
<th>Non-CVD Mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR (95% CI)</td>
<td>$P$ Value</td>
<td>HR (95% CI)</td>
</tr>
<tr>
<td>All</td>
<td>1.25 (0.96–1.64)</td>
<td>0.102</td>
<td>1.61 (1.03–2.53)</td>
</tr>
<tr>
<td></td>
<td>0.98 (0.68–1.41)</td>
<td>0.922</td>
<td>1.10 (0.63–1.92)</td>
</tr>
<tr>
<td></td>
<td>1.63 (1.03–2.56)</td>
<td>0.036</td>
<td>4.86 (1.73–13.66)</td>
</tr>
<tr>
<td>Men</td>
<td>1.23 (0.94–1.61)</td>
<td>0.138</td>
<td>1.57 (1.00–2.49)</td>
</tr>
<tr>
<td></td>
<td>0.99 (0.69–1.42)</td>
<td>0.961</td>
<td>1.10 (0.62–1.93)</td>
</tr>
<tr>
<td></td>
<td>1.62 (1.02–2.59)</td>
<td>0.042</td>
<td>4.03 (1.32–12.26)</td>
</tr>
<tr>
<td></td>
<td>1.29 (0.97–1.72)</td>
<td>0.076</td>
<td>1.72 (1.06–2.79)</td>
</tr>
<tr>
<td></td>
<td>0.96 (0.65–1.41)</td>
<td>0.828</td>
<td>1.12 (0.61–2.05)</td>
</tr>
<tr>
<td></td>
<td>1.90 (1.16–3.11)</td>
<td>0.011</td>
<td>6.51 (1.78–23.79)</td>
</tr>
<tr>
<td>Women</td>
<td>1.14 (0.78–1.66)</td>
<td>0.507</td>
<td>0.99 (0.59–1.68)</td>
</tr>
<tr>
<td></td>
<td>1.33 (0.76–2.30)</td>
<td>0.315</td>
<td>1.38 (0.79–2.43)</td>
</tr>
<tr>
<td></td>
<td>1.72 (1.06–2.79)</td>
<td>0.421</td>
<td>1.02 (0.60–1.72)</td>
</tr>
<tr>
<td></td>
<td>1.38 (0.79–2.43)</td>
<td>0.257</td>
<td>1.19 (0.79–1.77)</td>
</tr>
<tr>
<td></td>
<td>0.89 (0.51–1.57)</td>
<td>0.695</td>
<td>1.56 (0.86–2.81)</td>
</tr>
</tbody>
</table>

The cutoff point is the sex-specific highest quartile of AGES ($9.9$ U/mL in men; $9.0$ U/mL in women). HR is from the Cox proportional hazards model (the highest quartile vs the lowest 3 quartiles).
oxidative stress in atherosclerosis because anti-AGE antibody recognizes oxidatively modified proteins as well as glucose-modified compounds. However, high AGE levels predicted mortality even after adjustment for hs-CRP, indicating that AGEs probably increase mortality, at least in part, independently of inflammation in the vascular wall.

Circulating AGEs predicted mortality only in women. Therefore, it is possible that AGEs represent an additional risk for CVD only in low-risk individuals (compared with men, women in this study had a lower frequency of smoking, higher levels of HDL cholesterol, a lower ratio of total cholesterol/HDL cholesterol, and low alcohol intake). Further studies are needed to substantiate this notion, including therapeutic studies with AGE-lowering agents. Lowering of dietary AGEs in short-term studies has been shown to reduce inflammatory mediators such as peripheral blood mononuclear cell tumor necrosis factor-α and hs-CRP,

Seminar levels of AGEs did not correlate with fasting plasma glucose in our study, perhaps reflecting that the anti-AGE antibody recognizes the more heavily modified glucose compounds as well as oxidatively modified proteins. Furthermore, because CML can be formed from glucose and lipid modification of proteins, this might also contribute to the lack of correlation with fasting plasma glucose because the antibody we used in the assay recognizes CML.

This study has limitations. In spite of a long follow-up period, the number of end points was relatively low, particularly in women. Furthermore, the relatively high interassay coefficient of variation might possibly underestimate the full etiologic contribution of increased serum levels of AGEs in atherosclerosis development. Long-term storage of serum samples at −20°C might influence the measured AGE serum levels. However, all samples were stored under identical conditions, and consequently, differences in AGE levels should represent differences initially present in the samples when they were drawn. It is not likely that the storage had a different effect on AGE levels among those who died compared with those who did not. Furthermore, the staff of the laboratory in Oslo, where all AGE measurements were done, was unaware of the mortality status of participants.

Increased AGE levels did not predict atherosclerotic events in men, which may be because of a high frequency of smoking and other risk factors in men. Other studies have shown increased serum AGE levels in smokers,

In summary, we demonstrate for the first time that high AGE serum levels predict total, CVD, and CHD mortality in nondiabetic women. Therefore, the measurement of serum AGEs might identify high-risk individuals in a low-risk population.

Acknowledgments

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References

30. World Health Organization. 1983 proposal for the multinational Moni-toring of Trends and Determinants in Cardiovascular Disease and


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