

# Circulation

**Increased Serum Levels of Advanced Glycation End Products Predict Total and  
Cardiovascular Mortality in Women with Type 2 Diabetes: a population-based 18-year  
follow-up study**

Bente K Kilhovd, Auni Juutilainen, Seppo Lehto, Tapani Rönnemaa, Peter A Torjesen, Kristian F  
Hanssen, and Markku Laakso  
CIRCULATIONAHA/2006/667774  
Due: 25 Oct 2006

**This information is current as of October 16, 2006**

Disclaimer: The contents of this manuscript are confidential and intended for review purposes only.  
Downloaded from <http://submit-circ.ahajournals.org> on October 16, 2006

## **Author Disclosures**

**Bente K Kilhovd:** No disclosures

**Auni Juutilainen:** No disclosures

**Seppo Lehto:** No disclosures

**Tapani Rönnemaa:** No disclosures

**Peter A Torjesen:** No disclosures

**Kristian F Hanssen:** No disclosures

**Markku Laakso:** No disclosures

# **INCREASED SERUM LEVELS OF ADVANCED GLYCATION END PRODUCTS PREDICT TOTAL AND CARDIOVASCULAR MORTALITY IN WOMEN WITH TYPE 2 DIABETES**

**A population-based 18-year follow-up study**

**Kilhovd: AGEs predict mortality in type 2 diabetic women**

Bente K. Kilhovd\*, Auni Juutilainen, Seppo Lehto, Tapani Rönnemaa, Peter A. Torjesen, Kristian F. Hanssen, Markku Laakso

From the Diabetes Research Centre – Aker and Ullevål University Hospitals and Department of Medicine (B.K.K, K.F.H.); Hormone Laboratory (P.A.T.), Aker University Hospital HF, Faculty of Medicine, University of Oslo, Oslo, Norway; Department of Medicine (A.J., S.L., M.L.) University of Kuopio and Kuopio University Hospital, Kuopio, and the Department of Medicine (T.R.) University of Turku, Finland

\*Present address: Asker and Bærum Hospital HF

## **Correspondence to:**

Markku Laakso  
Academy Professor  
Department of Medicine  
University of Kuopio  
70210 Kuopio, Finland  
Phone +358-17-172151

E-mail [markku.laakso@kuh.fi](mailto:markku.laakso@kuh.fi)

**The total word count: 5095**

The research was supported by grants from the Norwegian Foundation for Health and Rehabilitation, Aker Diabetes Research Fund and the Norwegian Diabetes Association, and by grants from the Finnish Diabetes Research Foundation and the Academy of Finland.

## ABSTRACT

**Background** – Advanced glycation end products (AGEs), modification products formed by glycation or glycoxidation of proteins and lipids, have been linked to premature atherosclerosis in patients with diabetes. We investigated whether increased serum levels of AGEs predict total, cardiovascular (CVD) or coronary heart disease (CHD) mortality in a population-based study.

**Methods and Results** – Serum levels of AGEs were determined by immunoassay in a random sample of 874 Finnish diabetic subjects (488 men, 386 women), aged 45 to 64 years. These subjects were followed for 18 years for all-cause mortality, CVD and CHD mortality.

Multivariate Cox regression model showed a significant association of serum levels of AGEs with all-cause ( $P=0.004$ ) and CVD mortality ( $P=0.042$ ) in women, but not in men. Serum levels of AGEs in the highest quartile predicted all-cause (hazards ratio [HR] 1.61; 95% confidence intervals [CI], 1.22 to 2.12;  $P=0.001$ ) and CVD (HR 1.42; 95% CI 1.01 to 2.00;  $P=0.046$ ) mortality in women, even after adjustment for confounding factors, including high-sensitive C-reactive protein. No statistically significant association between AGEs and CHD was found.

**Conclusions** – Increased serum levels of AGEs predict total and CVD mortality in women with type 2 diabetes.

**Key Words:** glycoproteins, cardiovascular diseases, coronary disease, diabetes mellitus, women

Advanced glycation end products (AGEs) are short- and long-term modification products of glycation or glycoxidation of proteins and lipids.<sup>1,2</sup> AGEs are a heterogeneous group of compounds with multiple biological effects, some of which are mediated by interacting with receptors, including RAGE (the receptor for AGE) on endothelial cells, smooth muscle cells and macrophages.<sup>3-5</sup> Furthermore, AGEs have been demonstrated in atherosclerotic plaques.<sup>6</sup> AGEs are thought to contribute to the development of atherosclerosis by activating the transcription factor NF- $\kappa$ B through RAGE binding, resulting in induction of cellular adhesion molecule expression and cytokine activation,<sup>7,8</sup> or through glycoxidation of lipoproteins and increased foam cell formation.<sup>9,10</sup> AGEs might also quench nitric oxide and mediate impaired endothelial function.<sup>11</sup>

Increased AGE modification of long-lived proteins such as collagen increases cross-linking and stiffening of arteries.<sup>12</sup> Experimental studies in animals, and in humans, have shown that cross-link breaker treatment results in greater vascular compliance.<sup>13,14</sup> In a cross-sectional study elevated levels of circulating AGEs have correlated with the extent of coronary artery occlusion in type 2 diabetic patients.<sup>15</sup> We recently reported that high serum levels of AGEs predict coronary heart disease (CHD) mortality in nondiabetic women, but not in nondiabetic men.<sup>16</sup>

No study has been undertaken to examine whether there is any relationship between serum levels of AGEs and the subsequent development of CVD in a prospective follow-up of a large number of diabetic subjects. Therefore, the aim of the present study was to investigate whether increased serum levels of AGEs predict total, CVD and CHD mortality in 874 individuals with type 2 diabetes.

## RESEARCH DESIGN AND METHODS

**Baseline study.** The study population included 1,059 subjects (581 men, 478 women) with type 2 diabetes, aged 45-64 years, born and living in Kuopio, eastern Finland or in Turku, western Finland. The formation of the study population has been described in detail previously.<sup>17</sup> Subjects with type 1 diabetes were excluded on the basis of the age of onset of diabetes, history of ketoacidosis, and glucagon-stimulated C-peptide measurements at

baseline. The present study included 874 subjects (488 men, 386 women) with type 2 diabetes whose serum samples were available for the measurement of AGEs.

The study protocol included one outpatient visit to the Clinical Research Unit of the University of Kuopio or the Rehabilitation Research Centre of the Social Insurance Institution in Turku, as previously described in detail.<sup>17</sup> The visit included an interview on the history of chest pain suggestive of CHD, smoking, alcohol intake, physical activity, and the use of drugs. All medical records of subjects who reported that they had been admitted to hospital for chest pain symptoms were reviewed. Review of the medical records was performed after a careful standardization of the methods between the reviewers in Kuopio (M.L.) and Turku (T.R.). The WHO criteria for verified definite or possible MI, based on chest pain symptoms, electrocardiogram (ECG) changes, and enzyme determinations, were used to define previous MI.<sup>18</sup>

Smoking status was based on an interview. In all statistical analyses, subjects were classified as non-smokers or current smokers. Blood pressure was measured in the sitting position after a 5-min rest with a mercury sphygmomanometer and read to the nearest 2 mmHg. Subjects were classified as having hypertension if they were receiving drug treatment for hypertension or if their systolic blood pressure was at least 160 mmHg or diastolic blood pressure at least 95 mmHg.

At baseline, 273 diabetic men (55.9%), and 269 diabetic women (69.7%) were hypertensive. Diabetes was treated with diet only in 71 men (14.5%) and 43 women (11.1%), with oral hypoglycaemic drugs but not with insulin in 358 men (73.4%) and 281 women (72.8%), and with insulin in 59 men (12.1%) and 61 women (15.8%).

All laboratory specimens were drawn at baseline after a 12-h fast at 0800. Serum levels of AGEs were measured with a competitive immunoassay developed in our laboratory.<sup>19</sup> Briefly, we used polyclonal anti-AGE antibodies from rabbit immunised with AGE-RNase. Europium labelled anti-rabbit IgG was used as an indicator, and AGE-bovine serum albumin (BSA) was used as standard. Triplicates of standard or sample together with a fixed amount of anti-AGE antibody were added to microtiter plate wells coated with AGE-BSA. The plates were incubated while shaking for two hours, washed, and then indicating 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 µg/ml AGE-BSA standard.<sup>20</sup> The serum concentrations of AGEs were adjusted for total protein concentration according to Berg.<sup>19</sup> Inter-assay 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 antibodies obtained from one animal were used in measurements. Because no significant differences were found between the results obtained in these batches, they were pooled for statistical analyses. No significant differences in baseline characteristics were found between participants included, i.e. those whose serum was or was not available for AGE determination, in the present study.

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 sulphate-MgCl<sub>2</sub>.<sup>21</sup> LDL cholesterol was calculated using the Friedewald formula.<sup>22</sup> Total protein concentration (inter-assay variation 4.5%) was measured with the Coomassie brilliant blue method (Bio-Rad). High-sensitive C-reactive protein (hs-CRP) was determined by latex turbidimetric immunoassay (Wako Chemicals). Analytical detection limit of the assay was 3.3% at the mean level of 1.5mg/L and 2.6% 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, hospital records and autopsy records were used, if available. The causes of death were reviewed by S.L. and A.J. The end points used in this study were all-cause death, non-CVD death, CVD death, and CHD death. CVD death was defined by codes 390 to 459 and CHD death by codes 410 to 414, based on the International Classification of Diseases 9<sup>th</sup> Revision.

**Statistical methods.** Data analyses were conducted with the SPSSX, SPCC/PC+ and SPSS 11.0.1 programs (SPSS). Results for continuous variables are given as means ± SD or percentages. The bivariate correlation of continuous variables with AGEs was assessed by Spearman's correlation coefficient. The differences among the groups were assessed by the  $\chi^2$

test or Student's two-tailed  $t$  test for independent samples, when appropriate. Univariate and multivariate Cox regression model and Kaplan-Meier survival curves with log rank test statistics were used to investigate the association of cardiovascular risk factors with total, non-CVD, CVD, and CHD mortality. In multivariate Cox models, the adjustment was done for age, area of residence, gender, body mass index (BMI), current smoking, hypertension (blood pressure  $\geq 160/95$  mm Hg or drug treatment for elevated blood pressure), total cholesterol, triglycerides, HDL cholesterol, and menopausal status (in women).

**Approval of Ethics Committee.** This study was approved by the Ethics Committee of Kuopio University Hospital and the Turku University Central Hospital. All study subjects gave informed consent.

The authors had full access to the data and take responsibility for its integrity. All authors have read and agree to the manuscript as written.

## RESULTS

During the 18-year follow-up period altogether 362 men and 281 women died, including 245 men and 189 women who died of cardiovascular causes. Compared to men, women were older, had higher BMI and more often hypertension, had higher levels of total and HDL cholesterol, total triglycerides, fasting plasma glucose, and HbA1 (Table 1). Men were more often smokers and had a history of previous myocardial infarction. Serum levels of AGEs were significantly higher in men than in women.

The prevalence of current smoking and the levels of total cholesterol, triglycerides and HDL cholesterol did not differ between the highest quartile vs. the other quartiles of AGEs. Serum AGE levels did not significantly correlate with age, fasting plasma glucose, HbA1, total cholesterol, HDL cholesterol, total triglycerides, or BMI (range of correlations from -0.034 to 0.053). Exclusion of 3 patients whose serum creatinine was  $\geq 200$   $\mu\text{mol/L}$  from statistical analyses did not change the results.

Table 2 gives unadjusted and multiple-adjusted hazard ratios (HRs) for serum levels of AGEs, as a continuous variable, as a predictor for all-cause, non-CVD, CVD and CHD mortality (Cox regression model). Because AGE levels and gender had a significant interaction in their



effects on total mortality ( $P=0.021$  for interaction), results are presented separately for men and women. Adjusted serum AGEs were significantly related to total ( $P=0.004$ ), non-CVD ( $P=0.037$ ) and CVD ( $P=0.042$ ) mortality in women, but not in men.

Gender-specific quartiles of AGEs were:  $<5.8$ ,  $5.9-7.9$ ,  $8.0-10.2$  and  $>10.3$  U/mL in men, and  $<5.2$ ,  $5.3-7.2$ ,  $7.3-9.5$  and  $>9.6$  U/mL in women. Total and CVD mortality per 1000 person-years during the 18-year follow-up is presented in Figure 1. The highest quartile of serum AGEs versus the 3 other quartiles predicted total ( $P<0.001$ ) and CVD mortality ( $P=0.027$ ) in women but not in men.

Compared to women belonging to the lowest three AGE quartiles, women belonging to the highest AGE quartile had a 1.6-fold ( $P=0.001$ ) increased mortality from all causes, and 1.4-fold ( $P=0.046$ ) increased CVD mortality, independently of confounding factors. High AGE levels were also significantly associated with increased mortality from noncardiovascular causes in women ( $P=0.003$ ) (Table 3). Additional adjustment for hs-CRP did not essentially change the HRs. Of the non-CVD deaths 27.4% were diabetes-related deaths in men and 34.8% in women. AGE levels did not predict CHD mortality.

In Kaplan –Meier survival analysis, total ( $P=0.001$ ) and CVD mortality ( $P=0.011$ ) were significantly higher in women with high levels of AGEs than in women with low levels of AGEs (as cut-off point the gender-specific median). In men, high AGE levels did not predict total or CVD mortality (Figure 2).

## DISCUSSION

The present study shows for the first time that increased serum levels of AGEs predict total and CVD mortality in women with type 2 diabetes, and that serum AGEs in the top quartile ( $>9.6$  U/ml) is an independent risk factor for total and CVD mortality in these subjects.

An increase in skin AGEs can predict progression of diabetic retinopathy and nephropathy in type 1 diabetes.<sup>23</sup> Compared to skin biopsies for measuring AGEs, serum samples are simple, and with standardisation they could become a useful clinical tool. Previous cross-sectional studies have demonstrated increased serum levels of AGEs in patients with type 2 diabetes

and cardiovascular disease.<sup>15,24</sup> Our study shows that high levels of serum AGEs predict total and cardiovascular mortality in type 2 diabetic subjects in a long-term follow-up.

Serum AGEs are associated with inflammation markers.<sup>25</sup> AGE-modified proteins are a heterogeneous group of compounds that are formed through glycation, glycooxidation, or through glycooxidation/lipoxidation from lipids as for example N<sup>ε</sup>-(carboxymethyl)lysine (CML).<sup>2,26</sup> The dominant AGE epitope for binding to the RAGE receptor is CML.<sup>27</sup> Through binding to the RAGE receptor, CML may induce the activation of NF-κB<sup>27</sup> and vascular cell adhesion molecule-1 expression,<sup>28</sup> which might contribute to the development of premature atherosclerosis, although one study reported that endotoxin-free albumin derived AGEs were not sufficient to induce RAGE-mediated inflammatory signals.<sup>29</sup> Circulating AGEs may arise from intracellular reactive glucose metabolites,<sup>30</sup> or they can be formed in the circulation.<sup>31</sup> Recent studies suggest that the diet could be a source of AGEs.<sup>32</sup> We measured serum AGEs with a polyclonal anti-AGE antibody in order to detect most of the circulating AGEs. The polyclonal anti-AGE antibody has previously been shown to recognize CML as a major antigenic AGE epitope.<sup>33</sup>

Animal studies have demonstrated a causal involvement of AGEs in the development of atherosclerosis. In diabetic apolipoprotein E (apoE)-null mice normal chow increased atherosclerosis while soluble RAGE, in addition to the chow, inhibited the development of atherosclerosis.<sup>34</sup> A similar finding has recently been reported in a model of type 2 diabetes where apo E -/- mice were bred with db/db mice who are obese and insulin resistant and prone to develop type 2 diabetes. Administration of soluble RAGE to these mice resulted in significantly reduced atherosclerosis development as well as reduction in VCAM-1, tissue factor and matrix metalloproteinase (MMP)-9 expression.<sup>35</sup> Treatment with soluble RAGE in apoE-null diabetic mice with already developed atherosclerosis has been shown to significantly reduce atherosclerotic lesion area and complexity.<sup>36</sup>

In the present study fasting serum levels of AGEs in the highest quartile (>9.6 U/ml) were an independent predictor of total and CVD mortality in women. In diabetic women, compared to diabetic men, a larger proportion of cardiovascular risk is due to diabetes itself.<sup>37</sup> In agreement with this notion elevated levels of AGEs particularly in women might reflect an increase in inflammation or oxidative stress in atherosclerosis since the anti-AGE antibody used recognizes the oxidatively modified proteins as well as the glucose modified compounds.

Because high AGE levels predicted mortality even after the adjustment for hs-CRP, our results indicate that AGEs increase mortality also independently of inflammation in the vascular wall, possibly through increased vascular stiffening and subsequent elevation in systolic blood pressure. Increased AGE levels did not predict atherosclerotic events in men. The gender difference in our results remains unexplained, but could be at least in part explained by high prevalence of smoking and other risk factors in men, which could mask this association.

Our results suggest that elevated levels of circulating AGEs represent an additional risk factor for atherosclerotic complications in diabetic women. Further studies including therapeutic studies with AGE lowering compounds are needed to substantiate this conclusion. Lowering of dietary AGEs in short term studies has been shown to reduce inflammatory mediators such as peripheral blood mononuclear cell tumor necrosis factor- $\alpha$  and hs-CRP,<sup>38</sup> as well as glycoxidized LDL.<sup>32</sup> High-sensitivity CRP is an independent risk factor for CHD mortality not only in nondiabetic subjects<sup>39</sup> but also in patients with type 2 diabetes.<sup>40</sup> This suggests that inflammation plays an important role in fatal CHD events also among this high-risk population. The mechanisms linking high serum levels of AGEs to increased risk of death from non-cardiovascular causes in women remain unexplained and need further studies.

The serum levels of AGEs did not correlate significantly with fasting plasma glucose or HbA1c in our study. This might reflect the fact that the anti-AGE antibody recognizes the more heavily modified glucose compounds as well as the oxidatively modified proteins. Furthermore, as CML can be formed both from glucose and lipid modification of proteins, this might also contribute to the lack of correlation with fasting plasma glucose as the antibody we used in the assay recognizes CML. Several other studies have also reported a lack of correlation between serum AGEs and plasma glucose or HbA<sub>1c</sub> using this antibody.<sup>25,</sup>

33

In the present study high serum levels of AGEs predicted non-CVD mortality in women with type 2 diabetes with a greater relative risk than it predicted CVD mortality. The absolute impact of AGEs was, however, greater on CVD than on non-CVD mortality taking into account the greater frequency of CVD deaths (60%) compared to non-CVD deaths (40%) among diabetic women.

There are limitations to the present study. The relative high inter-assay coefficient of variation might possibly underestimate the full etiological contribution of increased serum levels of AGEs in atherosclerosis development. Furthermore, the long-term storage of serum samples might influence 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 unlikely that the storage had different effect on serum AGE levels among those who died compared with those who did not. The analytic work was performed by staff unaware of the mortality status of the participants.

In summary, we have shown for the first time that high serum levels of AGEs predict total and CVD mortality in women with type 2 diabetes. Therefore, the measurement of serum AGEs might identify individuals at high risk of cardiovascular complications among diabetic women.

## **Acknowledgements**

Prof. Rick Bucala, Picower Research Institute, Manhasset N. Y. kindly supplied the anti-AGE antibodies. We thank Ms. Turi Arnesen Siegwarth for skilful technical assistance.

Dr. Kilhovd was supported by a research grant from the EXTRA funds from the Norwegian Foundation for Health and Rehabilitation, and by the Diabetes Research Centre, Aker and Ullevål University Hospitals

## References

1. Brownlee M. Advanced protein glycosylation in diabetes and aging. *Annu Rev Med.* 1995;46:223-234.
2. Fu MX, Requena JR, Jenkins AJ, Lyons TJ, Baynes JW, Thorpe SR. The advanced glycation end product, Nepsilon-(carboxymethyl)lysine, is a product of both lipid peroxidation and glycoxidation reactions. *J Biol Chem.* 1996;271:9982-9986.
3. Schmidt AM, Vianna M, Gerlach M, Brett J, Ryan J, Kao J, Esposito C, Hegarty H, Hurley W, Clauss M. Isolation and characterization of two binding proteins for advanced glycosylation end products from bovine lung which are present on the endothelial cell surface. *J Biol Chem.* 1992;267:14987-14997.
4. Vlassara H, Li YM, Imani F, Wojciechowicz D, Yang Z, Liu FT, Cerami A. Identification of galectin-3 as a high-affinity binding protein for advanced glycation end products (AGE): a new member of the AGE-receptor complex. *Mol Med.* 1995;1:634-646.
5. Li YM, Mitsuhashi T, Wojciechowicz D, Shimizu N, Li J, Stitt A, He C, Banerjee D, Vlassara H. Molecular identity and cellular distribution of advanced glycation endproduct receptors: relationship of p60 to OST-48 and p90 to 80K-H membrane proteins. *Proc Natl Acad Sci U S A.* 1996;93:11047-11052.
6. Nakamura Y, Horii Y, Nishino T, Shiiki H, Sakaguchi Y, Kagoshima T, Dohi K, Makita Z, Vlassara H, Bucala R. Immunohistochemical localization of advanced glycosylation end products in coronary atheroma and cardiac tissue in diabetes mellitus. *Am J Pathol.* 1993;143:1649-1656.
7. Schmidt AM, Hori O, Chen JX, Li JF, Crandall J, Zhang J, Cao R, Yan SD, Brett J, Stern D. Advanced glycation endproducts interacting with their endothelial receptor induce expression of vascular cell adhesion molecule-1 (VCAM-1) in cultured human endothelial cells and in mice. A potential mechanism for the accelerated vasculopathy of diabetes. *J Clin Invest.* 1995;96:1395-1403.
8. Vlassara H, Fuh H, Donnelly T, Cybulsky M. Advanced glycation endproducts promote adhesion molecule (VCAM-1, ICAM-1) expression and atheroma formation in normal rabbits. *Mol Med.* 1995;1:447-456.
9. Bucala R, Makita Z, Koschinsky T, Cerami A, Vlassara H. Lipid advanced glycosylation: pathway for lipid oxidation in vivo. *Proc Natl Acad Sci U S A.* 1993;90:6434-6438.
10. Lyons TJ, Jenkins AJ. Lipoprotein glycation and its metabolic consequences. *Curr Opin Lipidol.* 1997;8:174-180.
11. Bucala R, Tracey KJ, Cerami A. Advanced glycosylation products quench nitric oxide and mediate defective endothelium-dependent vasodilatation in experimental diabetes. *J Clin Invest.* 1991;87:432-438.
12. Sims TJ, Rasmussen LM, Oxlund H, Bailey AJ. The role of glycation cross-links in diabetic vascular stiffening. *Diabetologia.* 1996;39:946-951.

13. Wolffenbuttel BH, Boulanger CM, Crijns FR, Huijberts MS, Poitevin P, Swennen GN, Vasan S, Egan JJ, Ulrich P, Cerami A, Levy BI. Breakers of advanced glycation end products restore large artery properties in experimental diabetes. *Proc Natl Acad Sci U S A*. 1998;95:4630-4634.
14. Kass DA, Shapiro EP, Kawaguchi M, Capriotti AR, Scuteri A, deGroof RC, Lakatta EG. Improved arterial compliance by a novel advanced glycation end-product crosslink breaker. *Circulation*. 2001;104:1464-1470.
15. Kiuchi K, Nejima J, Takano T, Ohta M, Hashimoto H. Increased serum concentrations of advanced glycation end products: a marker of coronary artery disease activity in type 2 diabetic patients. *Heart*. 2001;85:87-91.
16. Kilhovd BK, Juutilainen A, Lehto S, Rönnekaa T, Torjesen PA, Birkeland KI, Berg TJ, Hanssen KF, Laakso M. 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. *Arterioscler Thromb Vasc Biol*. 2005;25:815-820.
17. Laakso M, Rönnekaa T, Pyörälä K, Kallio V, Puukka P, Penttilä I. Atherosclerotic vascular disease and its risk factors in non-insulin-dependent diabetic and nondiabetic subjects in Finland. *Diabetes Care*. 1988;11:449-463.
18. World Health Organization. Proposal for the Multinational Monitoring of Trends and Determinants in Cardiovascular Disease and Protocol (MONICA Project). Geneva: World Health Organization, WHO/MNC/82.1 Rev. 1, Geneva, 1983.
19. Berg TJ, Bangstad HJ, Torjesen PA, Østerby R, Bucala R, Hanssen KF. Advanced glycation end products in serum predict changes in the kidney morphology of patients with insulin-dependent diabetes mellitus. *Metabolism*. 1997;46:661-665.
20. Makita Z, Vlassara H, Cerami A, Bucala R. Immunochemical detection of advanced glycosylation end products in vivo. *J Biol Chem*. 1992;267:5133-5138.
21. Kostner GM. Letter: Enzymatic determination of cholesterol in high-density lipoprotein fractions prepared by polyanion precipitation. *Clin Chem*. 1976;22:695.
22. Friedewald WT, Levy RI, Fredrickson DS. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. *Clin Chem*. 1972;18:499-502.
23. Genuth S, Sun W, Cleary P, Sell DR, Dahms W, Malone J, Sivitz W, Monnier VM. Glycation and carboxymethyllysine levels in skin collagen predict the risk of future 10-year progression of diabetic retinopathy and nephropathy in the diabetes control and complications trial and epidemiology of diabetes interventions and complications participants with type 1 diabetes. *Diabetes*. 2005;54:3103-3111.
24. Kilhovd BK, Berg TJ, Birkeland KI, Thorsby P, Hanssen KF. Serum levels of advanced glycation end products are increased in patients with type 2 diabetes and coronary heart disease. *Diabetes Care*. 1999;22:1543-1548.

25. Tan KC, Chow WS, Tam S, Bucala R, Betteridge J. Association between acute-phase reactants and advanced glycation end products in type 2 diabetes. *Diabetes Care*. 2004;27:223-228.
26. Reddy S, Bichler J, Wells-Knecht KJ, Thorpe SR, Baynes JW. N epsilon-(carboxymethyl)lysine is a dominant advanced glycation end product (AGE) antigen in tissue proteins. *Biochemistry*. 1995;34:10872-10878.
27. Kislinger T, Fu C, Huber B, Qu W, Taguchi A, Du Yan S, Hofmann M, Yan SF, Pischetsrieder M, Stern D, Schmidt AM. N(epsilon)-(carboxymethyl)lysine adducts of proteins are ligands for receptor for advanced glycation end products that activate cell signaling pathways and modulate gene expression. *J Biol Chem*. 1999;274:31740-31749.
28. Boulanger E, Wautier MP, Wautier JL, Boval B, Panis Y, Wernert N, Danze PM, Dequiedt P. AGEs bind to mesothelial cells via RAGE and stimulate VCAM-1 expression. *Kidney Int*. 2002;61:148-156.
29. Valencia JV, Mone M, Koehne C, Rediske J, Hughes TE. Binding of receptor for advanced glycation end products (RAGE) ligands is not sufficient to induce inflammatory signals: lack of activity of endotoxin-free albumin-derived advanced glycation end products. *Diabetologia*. 2004;47:844-852.
30. Brownlee M. Biochemistry and molecular cell biology of diabetic complications. *Nature*. 2001;414:813-820.
31. Bucala R, Makita Z, Vega G, Grundy S, Koschinsky T, Cerami A, Vlassara H. Modification of low density lipoprotein by advanced glycation end products contributes to the dyslipidemia of diabetes and renal insufficiency. *Proc Natl Acad Sci U S A*. 1994;91:9441-9445.
32. Cai W, He JC, Zhu L, Peppas M, Lu C, Uribarri J, Vlassara H. High levels of dietary advanced glycation end products transform low-density lipoprotein into a potent redox-sensitive mitogen-activated protein kinase stimulant in diabetic patients. *Circulation*. 2004;110:285-291.
33. Berg TJ, Clausen JT, Torjesen PA, Dahl-Jørgensen K, Bangstad HJ, Hanssen KF. The advanced glycation end product Nepsilon-(carboxymethyl)lysine is increased in serum from children and adolescents with type 1 diabetes. *Diabetes Care*. 1998;21:1997-2002.
34. Park L, Raman KG, Lee KJ, Lu Y, Ferran LJ, Jr., Chow WS, Stern D, Schmidt AM. Suppression of accelerated diabetic atherosclerosis by the soluble receptor for advanced glycation endproducts. *Nat Med*. 1998;4:1025-1031.
35. Wendt T, Harja E, Bucciarelli L, Qu W, Lu Y, Rong LL, Jenkins DG, Stein G, Schmidt AM, Yan SF. RAGE modulates vascular inflammation and atherosclerosis in a murine model of type 2 diabetes. *Atherosclerosis*. 2006;185:70-77.
36. Bucciarelli LG, Wendt T, Qu W, Lu Y, Lalla E, Rong LL, Goova MT, Moser B, Kislinger T, Lee DC, Kashyap Y, Stern DM, Schmidt AM. RAGE blockade stabilizes established atherosclerosis in diabetic apolipoprotein E-null mice. *Circulation*. 2002;106:2827-2835.
37. Juutilainen A, Kortelainen S, Lehto S, Rönkä T, Pyörälä K, Laakso M. Gender difference in the impact of type 2 diabetes on coronary heart disease risk. *Diabetes Care*. 2004;27:2898-2904.



38. Vlassara H, Cai W, Crandall J, Goldberg T, Oberstein R, Dardaine V, Peppas M, Rayfield EJ. Inflammatory mediators are induced by dietary glycoxidants, a major risk factor for diabetic angiopathy. *Proc Natl Acad Sci U S A*. 2002;99:15596-15601.
39. Kuller LH, Tracy RP, Shaten J, Meilahn EN. Relation of C-reactive protein and coronary heart disease in the MRFIT nested case-control study. Multiple Risk Factor Intervention Trial. *Am J Epidemiol*. 1996;144:537-547.
40. Soinio M, Marniemi J, Laakso M, Lehto S, Rönkämaa T. High-sensitivity C-reactive protein and coronary heart disease mortality in patients with type 2 diabetes: a 7-year follow-up study. *Diabetes Care*. 2006;29:329-333.

## FIGURE LEGENDS

**Figure 1.** Total and CVD mortality per 1000 person years during the 18-year follow-up in study patients stratified by AGE quartiles. P-value denotes the difference of Q4 to Q1-Q3.

**Figure 2.** Kaplan-Meier estimates of survival for total and cardiovascular disease (CVD) mortality according to the gender-specific medians of advanced glycation end products (AGEs) in 488 men and in 386 women with type 2 diabetes during the 18-year follow-up. P-values are from log rank-tests. The darker lines denote subjects with AGEs above and the lighter lines with small dots denote subjects below the gender-specific median.

**Table 1.** Baseline characteristics (N, %, or mean  $\pm$  SD)

	Men	Women	<i>p</i> -value
N	488	386	
Age (years)	57.6 $\pm$ 4.9	59.0 $\pm$ 4.9	<0.001
BMI (kg/m <sup>2</sup> )	28.3 $\pm$ 4.4	30.3 $\pm$ 5.7	<0.001
Current smoking (%)	25	6	<0.001
Hypertension (%)	56	70	<0.001
Previous myocardial infarction (%)	20	12	0.001
Total cholesterol (mmol/l)	6.4 $\pm$ 1.4	7.1 $\pm$ 2.0	<0.001
High-density lipoprotein cholesterol (mmol/l)	1.16 $\pm$ 0.33	1.27 $\pm$ 0.38	<0.001
Total / HDL cholesterol ratio	6.0 $\pm$ 2.1	6.1 $\pm$ 3.2	0.345
Triglycerides (mmol/l)*	2.4 $\pm$ 1.9	2.9 $\pm$ 3.6	0.001
LDL cholesterol (mmol/l)	4.3 $\pm$ 1.2	4.6 $\pm$ 1.3	<0.001
Fasting plasma glucose (mmol/l)	11.4 $\pm$ 3.8	12.5 $\pm$ 4.1	<0.001
Glycated haemoglobin A1 (%)	9.7 $\pm$ 2.3	10.1 $\pm$ 1.9	0.010
Duration of diabetes	8.1 $\pm$ 4.2	8.0 $\pm$ 3.9	0.831
Total protein-adjusted AGE, U/ml	8.4 $\pm$ 4.1	7.7 $\pm$ 3.7	0.015

\*difference in means tested after logarithmic transformation

**Table 2.** Adjusted hazard ratios (95% CIs) from Cox proportional hazards model for AGEs (per increment of 1 U/ml) in relation to total, non-CVD, CVD, and CHD mortality during 18-year follow-up in 488 men and 386 women with type 2 diabetes

	HR* (95% CI)	p-value
<b><i>Total mortality</i></b>		
All	1.02 (1.00 - 1.04)	0.078
Men	1.00 (0.97 - 1.02)	0.725
Women	1.05 (1.02 - 1.08)	0.004
<b><i>Non-CVD mortality</i></b>		
All	1.01 (0.97 - 1.04)	0.749
Men	0.98 (0.92 - 1.03)	0.362
Women	1.05 (1.00 - 1.10)	0.037
<b><i>CVD mortality</i></b>		
All	1.02 (1.00 - 1.05)	0.061
Men	1.00 (0.97 - 1.03)	0.848
Women	1.04 (1.00 - 1.08)	0.042
<b><i>CHD mortality</i></b>		
All	1.02 (0.99 - 1.04)	0.263
Men	1.00 (0.96 - 1.03)	0.916
Women	1.03 (0.98 - 1.08)	0.253

\*Adjusted for age, area of residence, gender (in all), body mass index, current smoking, hypertension, total cholesterol, high-density lipoprotein cholesterol, triglycerides, and for menopausal status (in women)

**Table 3.** High serum levels (the gender-specific highest quartile range vs. the three lowest quartile ranges: cutpoints  $\geq 10.3$  mU/l in diabetic men and  $\geq 9.6$  mU/l in diabetic women) of AGEs as a risk factor for total, non-CVD, CVD and CHD mortality in diabetic subjects during 18-year follow-up in two Cox models with varying levels of adjustment

<i>p</i> -value	Model 1		Model 2	
	HR (95% CI)	<i>p</i> -value	HR (95% CI)	<i>p</i> -value
<b><i>Total mortality</i></b>				
All	1.14 (0.95 - 1.36)	0.160	1.18 (0.98 - 1.43)	0.077
Men	0.85 (0.66 - 1.09)	0.197	0.89 (0.69 - 1.16)	0.399
Women	1.61 (1.24 - 2.11)	<0.001	1.61 (1.22 - 2.12)	0.001
<b><i>Non-CVD mortality</i></b>				
All	1.05 (0.76 - 1.45)	0.757	1.16 (0.83 - 1.63)	0.382
Men	0.66 (0.41 - 1.07)	0.091	0.71 (0.42 - 1.19)	0.189
Women	1.99 (1.27 - 3.13)	0.003	2.11 (1.32 - 3.37)	0.002
<b><i>CVD mortality</i></b>				
All	1.18 (0.95 - 1.46)	0.141	1.19 (0.95 - 1.49)	0.129
Men	0.94 (0.70 - 1.26)	0.678	0.98 (0.72 - 1.32)	0.876
Women	1.45 (1.04 - 2.02)	0.027	1.42 (1.01 - 2.00)	0.046
<b><i>CHD mortality</i></b>				
All	1.13 (0.87 - 1.45)	0.361	1.14 (0.87 - 1.49)	0.332
Men	0.85 (0.61 - 1.19)	0.352	0.87 (0.62 - 1.24)	0.449
Women	1.48 (0.99 - 2.22)	0.055	1.51 (1.00 - 2.28)	0.052

Adjusted in Model 1: for age, area of residence, gender (in all), total cholesterol, current smoking, hypertension, body mass index, HDL cholesterol, triglycerides, and postmenopausal status (in women); in Model 2: as in Model 1 and additionally for CRP.

Figure 1

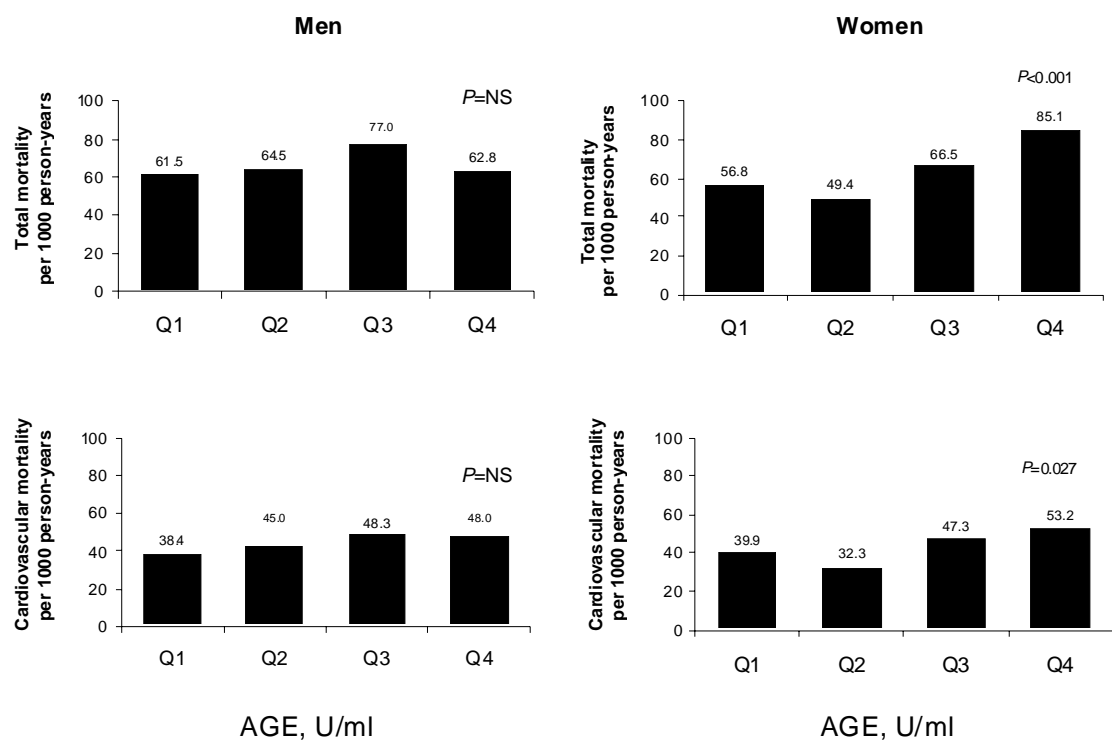


Figure 2

