

*Originals***Risk factors for mortality in Type II (non-insulin-dependent) diabetes: evidence of a role for neuropathy and a protective effect of HLA-DR4**

C.M. Forsblom<sup>1</sup>, T. Sane<sup>2</sup>, P.-H. Groop<sup>3</sup>, K.J. Tötterman<sup>1</sup>, M. Kallio<sup>4</sup>, C. Saloranta<sup>1</sup>, L. Laasonen<sup>5</sup>, P. Summanen<sup>6</sup>, M. Lepäntalo<sup>4</sup>, L. Laatikainen<sup>6</sup>, E. Matikainen<sup>7</sup>, A.-M. Teppo<sup>3</sup>, S. Koskimies<sup>8</sup>, L. Groop<sup>9</sup>

<sup>1</sup> Department of Medicine, Division of Internal Medicine, Helsinki University Hospital, Helsinki, Finland

<sup>2</sup> Division of Endocrinology, Helsinki University Hospital, Helsinki, Finland

<sup>3</sup> Division of Nephrology, Helsinki University Hospital, Helsinki, Finland

<sup>4</sup> Fourth Department of Surgery, Surgical Hospital, Helsinki, Finland

<sup>5</sup> Department of Radiology, Helsinki University Hospital, Helsinki, Finland

<sup>6</sup> Department of Ophthalmology, Helsinki University Hospital, Helsinki, Finland

<sup>7</sup> Finnish Institute of Occupational Health, Helsinki, Finland

<sup>8</sup> Finnish Red Cross Blood Transfusion Service, Helsinki, Finland

<sup>9</sup> Department of Endocrinology, University of Lund, Malmö, Sweden

**Summary** To test the hypothesis that interaction between genetic, immunological, clinical and metabolic risk factors influences the outcome of Type II (non-insulin-dependent) diabetes mellitus, we examined which of the above factors present at baseline were associated with mortality in 134 Type II diabetic patients followed for 9 years. Thirty-eight patients (29%) died during the follow-up period; the majority of whom (68%) died from cardiovascular disease. At baseline, the deceased patients had higher HbA<sub>1c</sub> values ( $p = 0.002$ ), higher LDL-triglycerides ( $p = 0.007$ ), lower HDL-cholesterol ( $p = 0.007$ ), higher non-esterified fatty acid (NEFA) concentrations ( $p = 0.014$ ), and higher albumin excretion rate ( $p < 0.0001$ ) than the patients who survived. In addition, the frequency of HLA-DR4 (21 vs 39%,  $p = 0.048$ ) and of parietal cell antibodies (5 vs 14%,  $p = 0.016$ ) were decreased in the deceased as compared to the living patients. Patients who died during follow-up also had more retinopathy (42 vs 16%,  $p = 0.002$ ), neuropathy (57 vs 23%,  $p < 0.001$ ), microalbuminuria (45 vs 6%,  $p < 0.0001$ ), coronary heart disease (50 vs 13%,  $p <$

0.0001), and peripheral vascular disease (27 vs 9%,  $p = 0.005$ ) at baseline than patients who survived. In a multiple logistic regression analysis macroangiopathy ( $p = 0.004$ ), neuropathy ( $p = 0.007$ ), HbA<sub>1c</sub> ( $p = 0.018$ ) and albumin excretion rate ( $p = 0.016$ ) were independent risk factors for death. In patients free of cardiovascular disease at baseline, conventional risk factors such as LDL-cholesterol ( $p = 0.005$ ) and age ( $p = 0.003$ ) were associated with subsequent development of cardiovascular disease. In conclusion, in addition to coexisting macroangiopathy, increased albumin excretion rate, poor glycaemic control and neuropathy are risk factors for cardiovascular mortality in patients with Type II diabetes. The presence of HLA-DR4 and signs of autoimmunity may be associated with decreased risk of cardiovascular disease. [Diabetologia (1998) 41: 1253–1262]

**Keywords** Type II (non-insulin-dependent) diabetes mellitus, HLA-DR4, microalbuminuria, mortality, cardiovascular risk factors, immunological markers, neuropathy, NEFA.

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*Corresponding author:* Dr. C. M. Forsblom, Helsinki University Hospital, Department of Medicine, Division of Internal Medicine, PL 346, FIN-00029 HYKS, Finland

*Abbreviations:* AER, Albumin excretion rate; BMI, body mass index; CHD, coronary heart disease; GAD, glutamic acid decarboxylase; NEFA, non-esterified fatty acids; PVD, peripheral vascular disease; HbA<sub>1c</sub>, glycated haemoglobin; PVR, pulse volume recording; WHO, World Health Organisation; LADA, latent autoimmune form of diabetes; MODY, maturity onset diabetes of the young.

The prognosis of patients with Type II (non-insulin-dependent) diabetes mellitus is primarily determined by the presence of macro- and microangiopathy. There is, however, a large variation in the propensity for the patient to develop these devastating complications. While poor glycaemic control and duration of diabetes are strong predictors of microangiopathy [1], their role as predictors of macroangiopathy is less clear [2–6]. In Type II diabetes, clustering of the metabolic syndrome (hyperglycaemia, obesity, hyper-

tension, hyperinsulinaemia, dyslipidaemia, and microalbuminuria) is associated with a particularly poor prognosis due to excess cardiovascular mortality [7]. However, only about 60% of Type II diabetic patients show features of the metabolic syndrome [8]. Among the remaining patients, about 10% have glutamic acid decarboxylase (GAD) antibodies and represent a latent autoimmune form of diabetes (LADA) [9, 10]. Genetic heterogeneity may further obscure the picture. The monogenic maturity onset diabetes of the young (MODY) forms are characterised by insulin deficiency, but normal insulin sensitivity [11] and less frequency of cardiovascular disease [12]. Increased prevalence of HLA-DR4 has been reported in Type II diabetic patients, but this increase was confined to patients with relative insulin deficiency [13]. In addition, HLA-DR4 has been shown to be associated with microangiopathy, particularly retinopathy in Type I (insulin-dependent) diabetic patients [14]. It is therefore likely that Type II diabetes includes a broad spectrum of disorders, in which disease heterogeneity influences susceptibility to diabetic complications.

To test the hypothesis that the interaction between clinical, genetic, immunological and metabolic risk factors influences the outcome of Type II diabetes, we examined which of the above factors present at baseline predicted mortality during a 9-year follow-up period. The design also allowed us to study the interaction between micro- and macroangiopathy as determinants of mortality in patients with Type II diabetes.

## Subjects and methods

### *Patients and study design*

We randomly selected 134 Type II diabetic patients, aged between 35 and 70 years, from the register of the Helsinki Diabetes Association. Type II diabetes was defined according to World Health Organisation (WHO) criteria [15]. Patients with malignancies or overt diabetic nephropathy ( $n = 3$ ) were excluded and the remaining 131 (67 male/64 female) patients participated in the study. All patients were first studied during the period 1983–1985, and all available patients ( $n = 86$ ) a second time during 1993–1995. Thirty-eight patients had died, while seven did not want to participate in the follow-up study. However, in most of these cases (4 patients) information about their current medical status could be obtained from their medical records. Information on causes of death was obtained from death certificates and medical files. The death certificates were reviewed by two of the authors independently of each other. All patients gave their informed consent and the study protocol was approved by the local ethics committee.

At baseline, all patients underwent a clinical examination with careful assessment of microangiopathy (retinopathy, nephropathy, and neuropathy) and macroangiopathy (cardiovascular and peripheral vascular disease). C-peptide and insulin concentrations were measured in serum samples taken before and 6 min after an intravenous injection of 1 mg of glucagon [16].

Insulin resistance was calculated as  $(\text{fasting insulin} \times \text{fasting glucose}) / (22.5)$ , using the homeostasis model assessment (HOMA) model [17]. Fasting blood samples were drawn for the measurements of blood glucose, glycated haemoglobin ( $\text{HbA}_{1c}$ ), serum total and lipoprotein lipids, and creatinine concentrations. In addition, organ-specific antibody (thyroid microsomal, thyroglobulin, islet-cell, and gastric parietal) concentrations were measured, and HLA-typing was performed. In a subset of patients ( $n = 64$ ) GAD antibodies were measured. Three 24-h urine collections were used for the measurement of urinary albumin excretion (AER) and creatinine clearance. At follow-up, all measurements except the assessment of retinopathy and neuropathy were repeated. However, instead of 24-h urine collections, overnight urine was collected for the measurement of AER.

### *Assessment of microangiopathy and macroangiopathy*

**Retinopathy.** The evaluation of the ocular fundi and the severity of retinopathy was assessed by direct ophthalmoscopy and fluorescein angiography. The findings were numerically evaluated with a scale from 0 to 5. Score 0 indicated no signs of retinopathy; 1 = minimal changes such as less than 10 microaneurysms per eye; 2 = slight background retinopathy with 10 or more microaneurysms per eye; 3 = moderate background retinopathy with plenty of microaneurysms, several small bleeding spots, lipid exudates and microinfarcts; 4 = maculopathy which indicated the same findings as in 3 but specifically in the macular region; 5 = proliferative retinopathy characterised by the same findings as in 3 but in addition presence of retinal neovascularisation. Retinopathy was considered present if a patient had 10 or more microaneurysms per eye in the fluorescein angiogram (scores 2–5).

**Neuropathy.** The findings of the clinical neurological examination were numerically evaluated with a scale from 0 to 12 (neuropathy score). The conduction velocities of peripheral nerves were measured with an electroneuromyography device (DISA Elektronik A/S, Skovlunde, Denmark). If two different nerves showed pathological conduction velocities the patient was considered to have neurophysiologically diagnosed neuropathy. To test the autonomic nervous system, heart rate variation at rest, during maximal 6 deep breaths per min, and during the Valsalva manoeuvre was recorded, and differences in heart rate and blood pressure between supine and postural positions were quantitated. The variation of heart rate was expressed as standard deviation (SD) of means and as the difference between maximum and minimum pulse frequency ( $\Delta\text{HR}$ ).

**Albuminuria.** Microalbuminuria was defined as AER 30–300 mg/24 h or 20–200  $\mu\text{g}/\text{min}$  (in overnight urine collections) and macroalbuminuria as AER greater than 300 mg/24 h or greater than 200  $\mu\text{g}/\text{min}$  in two out of three urine collections.

**Macrovascular disease.** Macroangiopathy was defined as the presence of coronary heart disease (CHD) and/or peripheral vascular disease (PVD) or stroke.

Electrocardiograms were recorded from each study subject and coded using the Minnesota Coding system by one investigator. CHD was defined as a medical history of myocardial infarction and/or the presence of probable myocardial infarction in the electrocardiogram (Minnesota codes 1.1–1.2), or if a patient was both treated with long-acting nitroglycerin and had signs of possible myocardial ischaemia in the electrocardiogram (Minnesota codes 1.3, 4.1–4.4, 5.1–5.3, 7.1)[18].

Blood pressure was measured in sitting position after a 15-min rest using a mercury sphygmomanometer. The mean value of three measurements obtained at 1-min intervals was calculated. Hypertension was defined as systolic blood pressure 160 mmHg or more and/or a diastolic blood pressure 95 mmHg or more or use of antihypertensive drugs.

Signs of heart failure were identified from a chest X-ray and the heart volume was estimated by an experienced radiologist. Heart volume was expressed as cc/m<sup>2</sup>.

**Peripheral vascular disease.** The non-invasive assessment of PVD included pulse volume recording (PVR), systolic blood pressure measurement at the ankle, calculation of ankle/arm systolic blood pressure ratio and measurement of great toe systolic blood pressure. PVR is a pneumoplethysmographic technique measuring volume changes in the extremity caused by a pulse wave [19]. Ankle blood pressure was measured using a 12-cm wide occluding cuff and Doppler device as a distal sensor [20]. Great toe systolic blood pressure was measured with a 24-mm wide occluding cuff and strain gauge as a distal sensor [21]. The diagnosis of PVD required two of the following criteria: (1) PVR amplitude at the ankle level 10 mm or less, (2) ankle/arm pressure ratio less than 0.85 and/or (3) great toe systolic blood pressure less than 50 mmHg.

**Assays.** Plasma glucose was assayed by a hexokinase method (Boehringer Mannheim, Mannheim, Germany). Glycohaemoglobin (HbA<sub>1c</sub>) concentration in blood was measured by micro-column cation exchange chromatography (Isolab, Akron, Ohio, USA) after overnight incubation of the samples in saline. The reference level for the assay was 5–7%. Serum insulin concentrations were measured in duplicate using antiserum M 8170, <sup>125</sup>I( Tyr A 19)-labelled porcine insulin as tracer and human monocomponent insulin as standard (Novo Research Institute, Bagsvaerd, Denmark) [22]. Precipitation of the antigen-antibody complex was performed using an ethanol separation technique. The sensitivity of the assay was 0.005 nmol/l, and the interassay coefficient of variation was 11%. C-peptide concentrations were determined in duplicate with an RIA method [23]. The sensitivity of the C-peptide assay was 0.01 nmol/l and the interassay coefficient of variation was 9%. Lipoprotein fractions were separated according to Havel et al. [24] by sequential ultracentrifugation from blood samples taken after an overnight (12 h) fast, using a Ti 50 rotor in Sorvall OTD 65 preparative ultracentrifuge (Du Pont Instruments, Wilmington, Del., USA) [25]. The concentrations of cholesterol and triglycerides in total plasma and in the separate lipoprotein fractions were determined by enzymatic methods using commercial kits (kit no. 187313 for cholesterol and kit no. 297771 for triglyceride, Boehringer Mannheim). Serum non-esterified fatty acids (NEFA) were measured with a microfluorometric method [26]. Creatinine in serum and urine was analysed with a kinetic method (Kone Oy Reag, Espoo, Finland). Urinary albumin excretion rate (AER) was measured with radioimmunoassay (RIA) from three 24-h urine collections at baseline, and with immunoturbidimetry from three overnight urine collections at follow-up. The detection limit for the RIA method was 2 mg/l, and the interassay coefficient of variation 5%. The corresponding values for the immunoturbidimetric method were 5 mg/l and 7.5 ± 1%, respectively. Organ-specific antibodies (thyroid microsomal antibodies, thyroglobulin antibodies, islet-cell antibodies, and gastric parietal antibodies) were determined as previously described [13]. GAD antibodies were measured by a radioimmunoprecipitation assay [27]. The limit for positivity was set to 18 U, which exceeded the mean + 3 SD for controls [9]. HLA typing was performed by a standard two-stage cytotoxicity method with a

**Table 1.** Causes of death during a 9-year follow-up in 38 type II diabetic patients

	<i>n</i>
Cardiovascular disease	26
– Myocardial infarction	19
– Heart failure	2
– Stroke	5
Uraemia	1
Infections	1
Neoplasms	3
Other causes <sup>a</sup>	5
Unknown	2
Total	38 (21 male/17 female)

<sup>a</sup> Other causes of death included: liver cirrhosis, drowning, suicide, intestinal occlusion, and lung embolism

total of 120 antisera defining 11 A-locus, 20 B-locus, and 7 Cw-locus specificities. Eight DR specificities, DR1-DRw8, were determined with a minimum of 60 antisera.

**Statistical analysis.** All data are expressed as mean ± SEM unless otherwise stated. Differences between group means were tested with analysis of variance (ANOVA), or covariance (ANCOVA) with age as a covariate, or Mann-Whitney test where appropriate, while Pearson's chi-square test or a two-tailed Fisher's test was used to test frequency differences. AER, creatinine clearance, and total triglyceride concentration were analysed after log<sub>e</sub> transformation due to positively skewed distributions. Independent risk factors for mortality and macroangiopathy were identified with backward stepwise multiple logistic regression analysis. All data were analysed using a BMDP statistical package (Biomedical Data Processing version 7.0, 1992, Los Angeles, Calif., USA) except the logistic regression analyses which were performed with NCSS statistical software (Biomedical Data Processing, version 6.0.21, 1996). A *p*-value less than 0.05 was considered statistically significant, except in the univariate comparisons of continuous variables where a *p*-value less than 0.02 was required.

## Results

### *Baseline characteristics of deceased and surviving patients*

Thirty-eight patients (29%) died during the follow-up. The majority (68%) of the deaths were due to cardiovascular disease (Table 1). Mortality was associated with higher HbA<sub>1c</sub>, and higher NEFA concentrations, lower HDL-cholesterol concentration, higher AER, and lower creatinine clearance at baseline (Table 2). In consequence, microalbuminuria was more prevalent in the deceased than in the living patients (45 vs 6%). In addition, patients who died had a higher prevalence of macroangiopathy (61 vs 22%) at baseline than patients who survived (Table 3). The difference in prevalence of cardiovascular disease at baseline was due to both cardiovascular and peripheral vascular disease. Of the patients con-

**Table 2.** Baseline clinical characteristics of Type II diabetic patients who died or survived during the 9-year follow-up period

	Deceased	Survived	<i>p</i> -value
<i>n</i> (male/female)	38 (21/17)	93 (46/47)	
Age (years)	59.5 ± 0.8	57.1 ± 0.6	
Duration of diabetes (years)	10.4 ± 0.8	8.8 ± 0.5	
Insulin treatment (%)	21	22	
Body mass index (kg/m <sup>2</sup> )	27.1 ± 0.6	27.2 ± 0.4	
Systolic BP (mmHg)	154 ± 4	154 ± 2	
Diastolic BP (mmHg)	85 ± 3	88 ± 1	
Fasting plasma glucose (mmol/l)	11.7 ± 0.6	10.6 ± 0.4	
HbA <sub>1c</sub> (%)	10.8 ± 0.3	9.7 ± 0.2	0.002
Triglycerides (mmol/l)	2.55 ± 0.29	2.26 ± 0.24	
VLDL-triglycerides (mmol/l)	1.64 ± 0.26	1.34 ± 0.17	
LDL-triglycerides (mmol/l)	0.60 ± 0.04	0.53 ± 0.04	0.007
Cholesterol (mmol/l)	6.22 ± 0.28	6.32 ± 0.14	
HDL-cholesterol (mmol/l)	1.24 ± 0.05	1.41 ± 0.04	0.007
HDL <sub>2</sub> -cholesterol (mmol/l)	0.64 ± 0.06	0.77 ± 0.04	0.013
HDL <sub>3</sub> -cholesterol (mmol/l)	0.59 ± 0.02	0.64 ± 0.01	
LDL-cholesterol (mmol/l)	4.40 ± 0.18	4.24 ± 0.11	
VLDL-cholesterol (mmol/l)	0.74 ± 0.12	0.63 ± 0.09	
NEFA (nmol/l)	991 ± 51	857 ± 35	0.014
Fasting serum-C-peptide (nmol/l)	0.55 ± 0.05	0.44 ± 0.04	
Fasting serum-insulin (nmol/l)	0.16 ± 0.01	0.14 ± 0.01	
HOMA insulin resistance	14.1 ± 1.8	10.8 ± 0.8	
Serum-creatinine (μmol/l)	95 ± 9	79 ± 2	
Creatinine clearance (ml · min <sup>-1</sup> · 1.73 m <sup>-2</sup> )	89 ± 4	108 ± 4	0.003
UAER (mg/24 h)	3.0 (1.0–78.0)	16.0 (1.0–300.0)	< 0.0001
Current smoking (%)	37	19	

All data are mean ± SEM except UAER median (range) with *p*-values adjusted for age

**Table 3.** Baseline characteristics of micro- and macrovascular disease in Type II diabetic patients who died or survived during the 9-year follow-up period

	Deceased ( <i>n</i> = 38)	Survived ( <i>n</i> = 93)	<i>p</i> -value
Microalbuminuria (%)	45	6	< 0.0001
Retinopathy (%)	42	16	0.002
Clinical neuropathy (%)	70	46	0.013
Neurophysiological neuropathy (%)	57	23	< 0.001
Valsalva ratio	1.42 ± 0.06	1.52 ± 0.03	0.002
Tilt ratio	1.25 ± 0.06	1.23 ± 0.01	
SD of heart rate at rest	41.3 ± 8.1	32.8 ± 1.7	
ΔHeart rate at deep breathing	20.4 ± 2.6	19.4 ± 1.1	
Macroangiopathy (%)	61	22	< 0.0001
Coronary heart disease (%)	50	13	< 0.0001
History of myocardial infarction (%)	18	6	0.038
History of stroke (%)	5	4	
ECG hard criteria (%)	24	5	0.002
Peripheral vascular disease (%)	27	9	0.006
Hypertension (%)	53	47	
Heart volume (cc/m <sup>2</sup> )	425 ± 17	397 ± 7	

All data are percentages or means ± SEM with *p*-values adjusted for age. SD = standard deviation of the mean, Δheart rate = difference between maximum and minimum pulse frequency

sidered to have CHD, only 46% fulfilled the Minnesota hard criteria for ECGs (Minnesota codes 1.1–1.2), while 42% had a verified history of myocardial infarction. In addition, retinopathy (42 vs 16%), clinical (70 vs 46%) and neurophysiologically defined neuropathy (57 vs 23%) were significantly more common among the deceased than the surviving patients. Likewise, autonomic neuropathy as indicated by a lower Valsalva ratio was more common in patients who died than in those who survived.

The HLA-DR4 genotype was more common in those who survived than in those who died (39 vs 21%). Otherwise, there were no significant differences in the frequencies of HLA-types between the two groups. There was an increased prevalence of parietal cell antibodies in the surviving vs deceased patients (14 vs 5%), otherwise the prevalence of organ-specific antibodies did not differ between the two groups (Table 4). In a subset of patients (*n* = 64) we also measured GAD antibodies. Only one of the patients

**Table 4.** Frequency (%) of HLA-DR and immunological markers in Type II diabetic patients who died or survived during the 9-year follow-up period

	Deceased (n = 38)	Survived (n = 93)	p-value
HLA-DR1	37	32	0.048
HLA-DR2	18	28	
HLA-DR3	32	36	
HLA-DR4	21	39	
HLA-DR3/DR4	47	63	
HLA-DR5	16	17	
HLA-DR6	10	14	
HLA-DR7	16	16	
HLA-DR8	13	5	0.016
Thyroglobulin antibodies	8	10	
Microsomal antibodies	27	19	
Islet cell antibodies	8	9	
Parietal cell antibodies	5	14	
GAD antibodies	7 (1 of 14 patients)	18 (9 of 50 patients)	

who died (7%) and nine patients who survived (18%) were GAD positive ( $p = \text{NS}$ ). None of the 16 patients with macroangiopathy at baseline was GAD positive compared with 21% (10 out of 48) of the patients without signs of macroangiopathy ( $p = 0.054$ ).

#### *Risk factors for mortality in multivariate analysis*

Independent risk factors for death were studied with backward stepwise multiple logistic regression analysis. The model included the variables which differed at baseline between deceased and surviving patients

(age, HbA<sub>1c</sub>, C-peptide, HDL-cholesterol, triglycerides, NEFA, creatinine clearance, AER, macroangiopathy, PVD, retinopathy, neurophysiological neuropathy, Valsalva ratio, HLA-DR4) together with gender. Of these, baseline AER, macroangiopathy, neuropathy, and glycaemic control were independently associated with death. (Table 5). Since lipids are known risk factors for macroangiopathy, we also tested whether lipids could be independent risk factors for mortality if macroangiopathy was removed from the model. In this analysis, AER, C-peptide, glycaemic control, and neuropathy were independent risk factors for death (Table 5). If microangiopathy was removed from the model glycaemic control and HDL-cholesterol were independent risk factors for death (Table 5). Finally, we tested whether microvascular disease (AER, retinopathy, and neuropathy) was independently associated with death in the absence of macroangiopathy and lipids. In all these models AER and neuropathy remained independent risk factors for death, while retinopathy was associated with death only in the absence of other microvascular complications in the model or if glycaemic control was removed from the model (Table 5).

*Variables associated with microangiopathy and macroangiopathy at baseline.* Since both microangiopathy (baseline AER and neuropathy) and macroangiopathy contributed to the mortality of the Type II diabetic patients, we examined which factors were independently associated with microangiopathy (retinopathy, neuropathy, and microalbuminuria) and macroangiopathy using multiple logistic regression analysis.

**Table 5.** Multiple logistic regression analysis with any mortality as dependent variable. Complete data were available from 113 patients

Variable	Coefficient	SEM	p-value
Model includes: Gender, age, HbA <sub>1c</sub> , ln(fasting C-peptide), HDL-cholesterol, ln(triglycerides), NEFA, ln(creatinine clearance), ln(AER), macroangiopathy at baseline, peripheral vascular disease, retinopathy, neurophysiological neuropathy, Valsalva ratio, and HLA-DR4.			
ln(AER)	0.467	0.194	0.016
Macroangiopathy	1.690	0.584	0.004
Neurophysiological neuropathy	1.662	0.618	0.007
HbA <sub>1c</sub>	0.362	0.153	0.018
ln(creatinine clearance)	-1.510	0.830	0.069
Fasting C-peptide	2.044	1.108	0.065
Model includes: Gender, age, HbA <sub>1c</sub> , ln(fasting C-peptide), HDL-cholesterol, ln(triglycerides), ln(AER), neurophysiological neuropathy, and retinopathy.			
ln(AER)	0.497	0.175	0.005
Fasting C-peptide	2.222	0.938	0.018
HbA <sub>1c</sub>	0.304	0.143	0.034
Neurophysiological neuropathy	1.179	0.522	0.024
Model includes: Gender, age, HbA <sub>1c</sub> , ln(triglycerides), and HDL-cholesterol.			
HbA <sub>1c</sub>	0.313	0.110	0.005
HDL-cholesterol	-1.751	0.738	0.018
Age	0.313	0.110	0.056
Model includes: Gender, age, ln(AER), retinopathy, and neurophysiological neuropathy.			
ln(AER)	0.581	0.173	0.001
Neurophysiological neuropathy	1.489	0.537	0.006
Retinopathy	0.169	0.545	0.032

**Table 6.** Clinical characteristics of Type II diabetic patients with no macroangiopathy at baseline who progressed or did not progress to macrovascular disease during the follow-up period

	Progressor	Non-progressor	<i>p</i> -value
<i>n</i> (male/female)	24 (15/9)	59 (29/30)	
Age (years)	58.9 ± 1.2	55.7 ± 0.8	0.020
Duration of diabetes (years)	9.5 ± 0.9	8.2 ± 0.6	
Insulin treatment (%)	29	22	
Body mass index (kg/m <sup>2</sup> )	27.4 ± 0.8	27.4 ± 0.5	
Systolic BP (mmHg)	149 ± 4	154 ± 3	
Diastolic BP (mmHg)	88 ± 2	88 ± 1	
Fasting plasma glucose (mmol/l)	10.4 ± 0.9	10.5 ± 0.4	
HbA <sub>1c</sub> (%)	10.1 ± 0.4	9.7 ± 0.2	
Triglycerides (mmol/l)	2.55 ± 0.52	2.17 ± 0.30	
VLDL-triglycerides (mmol/l)	1.51 ± 0.39	1.28 ± 0.21	
LDL-triglycerides (mmol/l)	0.54 ± 0.04	0.54 ± 0.06	
Cholesterol (mmol/l)	6.95 ± 0.32	6.10 ± 0.18	
HDL-cholesterol (mmol/l)	1.34 ± 0.07	1.46 ± 0.05	
HDL <sub>2</sub> -cholesterol (mmol/l)	0.72 ± 0.07	0.82 ± 0.05	
HDL <sub>3</sub> -cholesterol (mmol/l)	0.62 ± 0.02	0.63 ± 0.02	
LDL-cholesterol (mmol/l)	4.80 ± 0.28	3.99 ± 0.12	0.012
VLDL-cholesterol (mmol/l)	0.74 ± 0.19	0.62 ± 0.11	
NEFA (nmol/l)	813 ± 61	881 ± 50	
Fasting serum C-peptide (nmol/l)	0.38 ± 0.05	0.47 ± 0.05	
Fasting serum insulin (nmol/l)	0.14 ± 0.02	0.14 ± 0.02	
HOMA insulin resistance	11.6 ± 2.6	10.6 ± 1.0	
Serum creatinine (μmol/l)	96 ± 13	78 ± 2	
Creatinine clearance (ml · min <sup>-1</sup> · 1.73 m <sup>-2</sup> )	108 ± 7	106 ± 5	
UAER (mg/24 h)	5.0 (1.0–125.0)	2.0 (1.0–79.0)	
AER progression (%)	57	25	0.009
Current smoking (%)	38	17	(0.043)
Microalbuminuria (%)	13	8	
Retinopathy (%)	25	21	
Clinical neuropathy (%)	63	40	
Neurophysiological neuropathy (%)	32	30	
Valsalva ratio	1.50 ± 0.06	1.55 ± 0.04	
Tilt ratio	1.20 ± 0.02	1.23 ± 0.02	
SD of heart rate at rest	34.6 ± 3.8	30.4 ± 1.9	
ΔHeart rate at deep breathing	20.4 ± 2.4	18.1 ± 1.2	
Hypertension (%)	38	49	
Heart volume (cc/m <sup>2</sup> )	384 ± 15	412 ± 8	

All continuous data are mean ± SEM except UAER median (range) with *p*-values adjusted for age

The model included age, gender, HbA<sub>1c</sub>, C-peptide, HDL-cholesterol, triglycerides, NEFA, creatinine clearance, and HLA-DR4. Retinopathy was independently associated with HbA<sub>1c</sub> (*p* = 0.0001) and age (*p* = 0.005), neurophysiologically assessed neuropathy was associated with male sex (*p* = 0.0001) and HbA<sub>1c</sub> (*p* = 0.0009), while the presence of microalbuminuria was associated with HbA<sub>1c</sub> (*p* = 0.036) and creatinine clearance (*p* = 0.036). Macroangiopathy was associated with older age (*p* = 0.002) and low HDL-cholesterol (*p* = 0.004) concentrations.

*Risk factors for cardiovascular events in patients free of cardiovascular disease at baseline.* It is possible that risk factors for cardiovascular morbidity and mortality differ depending upon whether cardiovascular disease develops early or late in the course of diabetes. In an analysis of a subgroup of patients free from cardiovascular disease at baseline, cardiovascular morbidity and mortality were associated with older age, higher LDL-cholesterol concentra-

**Table 7.** Multiple logistic regression analysis with cardiovascular events in Type II diabetic patients with no macroangiopathy at baseline as dependent variable. Complete data were available from 79 patients

Variable	Coefficient	SEM	<i>p</i> -value
LDL-cholesterol	0.969	0.322	0.003
Age	0.181	0.065	0.005
HbA <sub>1c</sub>	0.353	0.173	0.041
HDL-cholesterol	-1.661	0.972	0.087

Model includes: Age, HbA<sub>1c</sub>, ln(fasting C-peptide), ln(triglycerides), LDL-cholesterol, HDL-cholesterol, NEFA, ln(AER), ln(creatinine clearance), retinopathy, neurophysiological neuropathy, Valsalva ratio, HLA-DR4, parietal cell antibodies, and history of smoking

tions, and smoking. In contrast to the findings in the total cohort, no difference was observed in glycaemic control or blood pressure between patients who developed or did not develop cardiovascular events. Although baseline AER did not differ between the two

groups, progression of AER from normoalbuminuria to microalbuminuria or macroalbuminuria was significantly more common in patients who developed cardiovascular disease during the follow-up period. (Table 6). These factors together with the risk factors operative in the total study (HbA<sub>1c</sub>, triglycerides, HDL-cholesterol, NEFA, C-peptide, creatinine clearance, AER, retinopathy, neuropathy, and Valsalva ratio) were then entered into a multiple logistic regression analysis. In this analysis, LDL-cholesterol, age, and HbA<sub>1c</sub> were risk factors for cardiovascular morbidity and mortality (Table 7).

## Discussion

This prospective study showed a total mortality of 29% during the 9-year follow-up period, cardiovascular events being the main cause of death. In a multiple logistic regression analysis mortality was associated with baseline glycaemic control, AER, neuropathy and macroangiopathy. However, the association between risk factors and outcome is not that simple. In the present study as well as in previous studies, Type II diabetes was considered as a homogenous disease entity. This is most likely not the case; Type II diabetes is rather a heterogeneous disorder which can be subdivided into subgroups based upon genetic and immunological markers [8]. Therefore, one has to consider the possibility that disease heterogeneity influences the development of complications.

Somewhat surprisingly, Type I diabetes susceptibility HLA genes, particularly HLA-DR4, were recently proposed to explain the majority of Type II diabetes in Finland [28]. The current finding of increased frequency of DR4 in patients who survived compared with deceased patients (39 vs 21%), may provide an explanation for these unexpected findings. Tuomilehto-Wolf et al. [28] reported a prevalence of HLA-DR4 of 57% in elderly Finnish men with Type II diabetes. The patients had an average age of 77 years, which is far beyond the life expectancy for a Finnish man with Type II diabetes. It is therefore likely, that the elderly men in the study by Tuomilehto-Wolf et al. represented a surviving Type II diabetic subgroup free of macrovascular disease [28]. The corollary of this finding is that the presence of Type I susceptibility genes in Type II diabetic patients seems to protect from macrovascular disease. In support of this, GAD antibody positive patients had a tendency to lower frequency of cardiovascular complications. The findings of increased prevalence of parietal cell antibodies in surviving Type II diabetic patients could also be related to an increase in either HLA-DR3 or HLA-DR4, which are known to be associated with autoimmune disease [29].

Although patients who survived had a higher prevalence of HLA-DR4 and parietal cell antibodies (and a tendency to higher prevalence of GAD antibodies) than patients who died, neither HLA-DR4 nor the presence of autoimmunity were associated with the outcome in the multivariate analysis. This could suggest that the (protective) effect of HLA is mediated through other mechanisms included in the analysis. HLA-DR4 and autoimmune markers are particularly increased in Type II diabetic patients with some degree of beta-cell dysfunction [13]. In contrast, high C-peptide concentrations most likely reflecting insulin resistance were also associated with mortality in the model without macroangiopathy (Table 5). Taken together with the reported long survival in Type II diabetic men with increased prevalence of HLA-DR4, the data suggest that HLA-DR4 identifies a subgroup of Type II diabetes characterised by low C-peptide, relatively normal insulin sensitivity, and reduced risk of cardiovascular disease.

The lipid profile in the patients who died was compatible with the lipid profile characteristic of the metabolic syndrome, i.e. increase in total triglyceride (both VLDL and LDL subfractions) and decrease in HDL-cholesterol (both HDL<sub>2</sub> and HDL<sub>3</sub> subfractions). Although not measured at baseline, increased amounts of atherogenic small dense LDL-cholesterol particles are known to be associated with these lipid abnormalities [30]. It can therefore be anticipated that the increase in the VLDL-particles partially reflected the presence of small dense LDL [31]. The patients who died during follow-up thereby fulfilled four of the criteria for the metabolic syndrome, i.e. high triglycerides, low HDL-cholesterol, insulin resistance (higher C-peptide) and microalbuminuria, whereas we could not find any evidence for higher blood pressure or obesity. Regarding obesity we have to make a reservation for abdominal obesity which was not assessed in the study. The presence of the metabolic syndrome is further supported by the elevated NEFA concentrations, since the metabolic syndrome has been associated with abnormalities in visceral fat lipolysis and elevated NEFA concentrations [32]. NEFA is a substrate for the production of atherogenic VLDL-triglycerides in the liver; in addition, elevated circulating NEFA levels could also lead to increased NEFA uptake and re-esterification in muscle. In support of this, insulin-resistant subjects have increased triglyceride concentrations in muscle [33, 34], and there is accumulating evidence for the existence of intracellular lipolysis [35] and fat oxidation in muscle [36]. An increased fat oxidation could then by substrate competition (Randle cycle) lead to decreased insulin-stimulated glucose metabolism [37].

The present study confirmed earlier prospective data on the predictive value of microalbuminuria for both total, and cardiovascular mortality in Caucasian populations [4,38–42]. Both all-cause (74 vs 19%)

and cardiovascular mortality (57 vs 21 %) were higher in microalbuminuric compared to normoalbuminuric Type II diabetic patients. In addition, AER predicted mortality in a multiple logistic regression analysis. Although microalbuminuria predicts diabetic nephropathy and thereby end-stage renal disease in Type II diabetic patients [43], increased mortality in end-stage renal disease cannot explain the increased total mortality in patients with microalbuminuria, since most Scandinavian Type II diabetic patients die before they develop overt renal disease [4,42]. In support of this, only 5 of the 86 surviving, restudied patients (6 %) developed diabetic nephropathy, and only 1 patient died of end-stage renal disease. Microalbuminuria in these patients may reflect generalised vascular dysfunction rather than actual renal disease [44]. Accordingly, Stehouwer et al. [45] have shown that microalbuminuria in Type II diabetes is associated with new cardiovascular events only in the presence of increased von Willebrand factor, a marker of endothelial dysfunction. Insulin resistance could also link microalbuminuria to macrovascular disease. We have earlier shown that microalbuminuria is associated with insulin resistance in both Type II diabetic patients and their non-diabetic relatives [46, 47]. Since insulin resistance is a risk factor for cardiovascular disease, it could be the common denominator for microalbuminuria and other cardiovascular risk factors characteristic of the insulin resistance syndrome [7].

In the present study, macroangiopathy and microangiopathy (neuropathy and AER) were independent risk factors for total mortality. In contrast, several known cardiovascular risk factors (triglycerides, HDL-cholesterol, blood pressure) did not predict mortality. If these variables were strongly associated with microangiopathy and/or macroangiopathy per se, this could remove them from the multiple logistic regression analysis. To test this hypothesis, we performed multiple logistic regression analyses using baseline microangiopathy (retinopathy, neuropathy, and microalbuminuria) and macroangiopathy as dependent variables. Microangiopathy, but not macroangiopathy, was associated with poor glycaemic control ( $HbA_{1c}$ ). However, glycaemic control was weakly associated with later cardiovascular events in patients free from such disease at baseline. Previous prospective studies have provided discrepant results regarding a role for glycaemic control in promoting macrovascular disease [3, 4, 38–40, 48]. In this prospective study, worse glycaemic control at baseline was associated with an increased mortality. Part of this effect of hyperglycaemia on mortality could be mediated through increased mortality associated with microvascular disease, especially nephropathy. Another possibility is that insulin-resistant patients had higher  $HbA_{1c}$  concentrations and that insulin resistance mediated the increased risk for cardiovascular disease.

Interestingly, in the present study, neuropathy, as assessed by neurophysiological methods, was an independent risk factor for all-cause mortality, which was not reflected by the clinical staging. It is obvious that the clinical diagnostic methods were too insensitive for this purpose, which could partly explain why neuropathy rarely is considered an independent risk factor for mortality. The autonomic nervous function was assessed using four different methods in order to increase the specificity of the diagnosis. However, only a lower Valsalva ratio was predictive of mortality in a univariate ( $p = 0.002$ ), but not in a multivariate analysis. To some extent, these data differ from those in a recent 10-year follow-up study of newly diagnosed Finnish Type II diabetic patients in which autonomic neuropathy was a strong predictor of cardiovascular mortality, even after adjustment for CHD, cardiovascular risk factors, and glycaemic control [49]. It is possible that the effect of autonomic neuropathy on mortality was attenuated by the effect of poor glycaemic control, a factor which was strongly associated with autonomic neuropathy in this study.

When we separately analysed patients without macroangiopathy at baseline conventional risk factors such as LDL-cholesterol and age were associated with the development of macrovascular disease. In this subgroup of patients baseline AER was not a risk factor for macroangiopathy. A potential explanation for this discrepancy is that those with macrovascular disease at baseline represented a high-risk group, the majority of whom had died during the follow-up. In these patients, the metabolic syndrome seems to be the strongest predictor of death. However, conventional risk factors such as high LDL-cholesterol concentrations are also operative, but this effect is not unmasked until the high-risk group has been omitted from the analysis.

In conclusion, mortality in Type II diabetic patients is primarily caused by macrovascular disease. In addition to pre-existing signs of macrovascular disease, poor glycaemic control, microalbuminuria, and neuropathy were independent risk factors for cardiovascular mortality. If patients had no macroangiopathy at baseline, conventional cardiovascular risk factors, such as LDL-cholesterol, were associated with cardiovascular morbidity also in Type II diabetes. Therefore, most risk factors are modifiable by intensified treatment, particularly of hyperglycaemia and dyslipidaemia.

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