The hyperglycemic milieu in diabetes results in the formation of advanced glycation end products (AGEs) that predominantly act through specific receptors, particularly the receptor for AGEs (RAGE). Two functional polymorphisms in the promoter of the RAGE gene (−429 T/C and −374 T/A) and one in the AGE binding domain in exon 3 (G82S) were studied in 996 Finnish type 1 diabetic patients. In patients with poor metabolic control (HbA1c >9.5%), the AA genotype of the −374 T/A polymorphism was more common in those with a normal albumin excretion rate than in those with proteinuria (30 vs. 10%, P = 0.01). We observed less coronary heart disease (6 vs. 14%, P < 0.05), acute myocardial infarction (2 vs. 14%, P = 0.01), and peripheral vascular disease (2 vs. 14%, P < 0.05) in patients with the AA genotype of the −374 T/A polymorphism than in those with the TT or TA genotype. Thus, the association between the RAGE −374 T/A homozygous AA genotype and cardiovascular disease as well as albumin excretion in type 1 diabetic patients with poor metabolic control suggests a gene-environment interaction in the development of diabetic nephropathy and cardiovascular complications. Diabetes 52:891–894, 2003

Elevated arterial blood pressure, proteinuria, progressive decline in renal function, and high cardiovascular morbidity and mortality are clinical characteristics of nephropathy in type 1 diabetic subjects (1,2). The chronic hyperglycemia associated with this condition results in formation and accumulation of irreversible advanced glycation end products (AGEs) that cause a plethora of adverse effects, resulting in dysfunction of the vasculature (3). These include changes in lipid metabolism, platelet function, and extracellular matrix composition (4), effects shown to be mediated by specific AGE binding receptors. The candidate receptor most likely to be involved in these processes is the receptor for AGEs (RAGE). Blockade of AGE/RAGE binding prevents the underlying cellular changes associated with vascular disease and the formation of atherosclerosis in animal models of diabetes (5,6). RAGE is normally expressed at low levels in the vasculature; however, studies of human subjects and animal models of diabetes reveal major upregulation of RAGE in the presence of vascular disease (7,8). The role for RAGE in diabetic renal disease is emphasized by animal models engineered to overexpress RAGE, which develop diabetic nephropathy more rapidly than their nontransgenic counterparts (9).

The role for a genetic influence on the pathogenesis is supported by studies that show familial clustering of diabetic nephropathy (10,11), although few genes have been identified as having a role in this disorder. The upregulation and pathogenic effects of RAGE in diabetic vascular disease highlight the RAGE gene as a candidate for involvement in the pathogenesis of nephropathy. The gene for RAGE is located on chromosome 6p21.3 near the HLA locus (12), and at least 30 polymorphisms have been identified (13–16). Most of the polymorphisms are rare coding changes or located in noncoding regions, including introns and the 5′ flanking region. Three polymorphisms have been highlighted in studies that include two common functional polymorphisms (−429 T/C and −374 T/A) in the promoter region and a common coding change of a Gly to Ser at amino acid 82. The −429 T/C and −374 T/A polymorphisms were shown to have a marked effect on transcriptional activity (14), with the G82S occurring in the AGE binding domain. Whether these functional polymorphisms play a role in the development of diabetic nephropathy or other complications in type 1 diabetes has not been established. Therefore, we studied these three polymorphisms in 996 type 1 diabetic patients with and without diabetic nephropathy.

The clinical characteristics of the four patient groups...
are shown in Table 1. In the entire cohort, the frequencies of the homozygous mutant genotype for the −429 T/C, −374 T/A, and G82S polymorphisms were 2, 13, and 1%, respectively. The −374 T/A and G82S polymorphisms were in Hardy-Weinberg equilibrium in all four groups, whereas the −429 T/C deviated significantly from equilibrium ($\chi^2 = 20.8, P < 0.0001$).

No difference in genotypes or allele frequencies was observed between the four groups. The AA genotype frequency of the −374 T/A polymorphism was 15% in patients with normoalbuminuria compared with 12% in those with proteinuria ($P = NS$). However, in patients with poor metabolic control, the AA genotype of the −374 T/A polymorphism was more common in subjects with normal albumin excretion than in those with proteinuria (Table 2). The HbA1c level did not affect the genotype frequencies of the 429 T/C and G82S polymorphisms (data not shown).

In the entire cohort, 106 patients had manifest cardiovascular disease (CVD). CVD was less common in those with the AA genotype than in those with the TT and TA genotypes of the −374 T/A polymorphism. In addition, patients with the AA genotype had less coronary heart disease (CHD) and acute myocardial infarction (AMI) (both included in the CVD variable) and less peripheral vascular disease (PVD) (not included in the CVD variable) (Table 3). Furthermore, the AA genotype of the −374 T/A polymorphism was associated with CVD in a multiple logistic regression analysis (Table 4).

The single nucleotide polymorphisms were in tight linkage disequilibrium with each other ($P < 0.0001$), but since we had no linkage phase information due to the study setting, we analyzed whether a specific genotype combination would associate with the analyzed phenotypes using the program MENDEL (17). Such an association would suggest a specific risk-increasing “haplotype block” (18) behind the findings, which could then contain the possible undetected causative variant. In this analysis by MENDEL option 12, the distributions of all the alleles as a set of three markers are analyzed together for each phenotype. The statistical significance of the difference in these distributions is tested using a Monte Carlo simulation approximation to Fisher’s exact test. Genotypes of the case and control subjects are permuted, and a distribution of the possible genotypes is generated. The program identifies the cell with the largest deviation in allele frequency from what is expected and tests the significance of this deviation with a permutation test. The alleles observed in both samples are included in the distributions under analysis. Phase-known haplotypes are not required to identify a difference in the distributions. The exact test allows for sparse and zero counts in cells of the distributions. No specific predisposing genotype combination was found, which suggests that the −374 T/A variation might be the actual causative variant and not in mere linkage disequilibrium with the actual variant.

Our results show an association between the −374 T/A AA genotype of the RAGE promoter and CVD as well as albumin excretion in type 1 diabetic patients with poor metabolic control. These results indicate that the AA genotype may have a protective role against some diabetic complications, particularly in those with poor metabolic control. Our results are, however, at conflict with a recent study by Poirier et al. (16) showing no association between diabetic nephropathy and the −374 T/A polymorphism. In that study, the sample size was smaller and the effect of poor glycemic control was not studied. Additionally, Poirier et al. showed a weak association between diabetic nephropathy and the −1,152 C/A polymorphism. However, recent data demonstrates that this is not a polymorphism but a gene-pseudogene difference (19). It is possible that our result is a type I statistical error. However, first, the A allele of the −374 T/A polymorphism alters transcription of the RAGE gene that, with evidence for involvement of RAGE in macrovascular disease, supports the existence of an association. Second, our a priori hypothesis postulated that any protective gene effect would be stronger in patients with a normal albumin excretion rate (AER) despite decades of poor metabolic control.
control. The results of the current study support the existence of a gene (RAGE)-environment (glycation) interaction that may explain some of the associations between poor glycemic control and development of nephropathy. Third, the risk of a false positive association is further diminished by the distribution of the −374 T/A genotypes in the patients with manifest CVD. We found less CVD in those with the AA genotype than in those with the T genotype. This potentially protective AA genotype was again predominantly present in those with metabolic control above the median HbA1c value (data not shown).

Furthermore, in analyzing the prevalence of PVD separately, there was, again, a significantly lower prevalence of the homozygous AA genotype in those with manifest PVD. Finally, the AA genotype of the −374 T/A polymorphism was independently associated with CVD in a multiple logistic regression analysis. Taken together, these results suggest that the −374 homozygous AA genotype is a marker for less atherosclerosis in type 1 diabetic patients.

Our results raise the question as to how altered transcription of the RAGE promoter region can affect susceptibility to CVD and proteinuria in type 1 diabetic patients. In such patients, the levels of AGEs are elevated in those with nephropathy and highest in patients with end-stage renal disease (ESRD) (20). Investigation of sites of AGE accumulation reveal, in both human and animal models of diabetes, that upregulation of RAGE occurs. Infusion of soluble RAGE, the extracellular truncated domain into diabetic mice engineered to develop accelerated atherosclerosis, prevents the development of disease of the vasculature (6). This implicates the regulation of RAGE as a crucial event in the pathogenesis of vascular disease in diabetes. Altered transcription induced by the −374 T/A polymorphism could therefore affect these processes, leading to a reduced level of RAGE and a different disease outcome.

In conclusion, the association between the RAGE −374 T/A homozygous AA genotype and CVD as well as albumin excretion in type 1 diabetic patients with poor metabolic control suggests a gene-environment interaction in the development of diabetic nephropathy and cardiovascular complications.

### RESEARCH DESIGN AND METHODS

**Subjects.** This study is part of the ongoing Finnish Diabetic Nephropathy (FinnDiane) Study, a comprehensive, multicenter, nationwide project with the aim to phenotype 25% of all adult patients with type 1 diabetes in Finland. The first 1,006 patients of the FinnDiane cohort with an ascertained renal status were recruited from 20 referral centers between 1994 and 1999. The patients were required to be C-peptide negative (<0.3 nmol/l) and to have permanent insulin treatment initiated before 35 years of age, within 1 year of diabetes diagnosis. Six patients with residual C-peptide secretion and four with evidence of nondiabetic kidney disease were excluded. The renal status was based on the AER in at least three consecutive overnight or 24-h urine collections. The patients with a normal AER (<20 μg/min or <30 mg/24 h, n = 321) were required to have a diabetes duration >15 years to ensure their renal status. A total of 166 patients had microalbuminuria (AER 20–200 μg/min or 30–300 mg/24 h), 325 proteinuria (AER >200 μg/min or >300 mg/24 h), and 184 ESRD. ESRD patients were either on dialysis (n = 44) or had a kidney transplant (n = 140). The years of renal replacement therapy initiation and diabetic nephropathy diagnosis were obtained from medical records. Information about CVD, including CHD, AMI, and stroke, was also obtained from medical records. All patients with PVD had bypass surgery performed on either leg. Information about CVD, AMI, antihypertensive treatment, or stroke in the parents was given by the proband using a questionnaire. Informed consent was obtained from all subjects participating in the study. The protocol followed the principles expressed in the Declaration of Helsinki and was approved by all local ethics committees.

**Assays.** Serum and urine creatinine levels for calculation of creatinine clearance were measured at a central laboratory by a modified Jaffé reaction using a Hitachi 911 E Automatic Analyzer (Boehringer Mannheim, Mannheim, Germany). Urinary albumin concentration was measured using radioimmunoassay (RIA) (Pharmacia, Upsala, Sweden) and serum C-peptide concentrations with a Human C-peptide RIA kit (Linco Research, St Charles, MO). HbA1c was measured using standardized assays in each referral center (normal range 4.0–6.0%). Serum HDL, LDL, and total cholesterol and triglycerides were analyzed using routine standardized laboratory methods at each center.

**Genotyping.** The polymorphisms of the RAGE gene were determined from DNA extracted from peripherally drawn blood samples. Genotyping, by an investigator unaware of the phenotypes, was performed using regular PCR. For the amplification of the region containing the −429 T/C and −374 T/A polymorphisms, we used previously described primers (14) with an added biotinylated F5 and 5′-CTGTGCTGTGAGGCTGCA-3′, respectively. We used the previously described G82S primers (13) with a biotinylated R5 end using a forward minisequencing primer 5′-TGCTGCGTCTCTCCATAAGC-3′. Linkage disequilibrium analyses were performed by the LinkDos and Genepop package (version 1.2).

**Statistical analysis.** Descriptive data are expressed as means ± SE or approved value (range), unless otherwise stated. Categorical variables were compared using the χ2 test. Normally distributed continuous variables were tested with Student’s t test, while nonnormally distributed variables were logistically transformed or assessed with the Mann-Whitney U test. To evaluate the independent association between categorical variables and CVD or diabetic nephropathy, a multiple logistic regression model was used with a means substitution of absent data. All statistical analyses were also performed separately for each sex, but since there were no differences between sexes, data were pooled. Analyses were carried out using a Statistica 4.1 statistical package (Statistica, Tulsa, OK). A P value <0.05 was considered statistically significant.

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RAGE POLYMORPHISMS AND DIABETIC NEPHROPATHY


