Lack of synergism between long-term poor glycaemic control and three gene polymorphisms of the renin angiotensin system on risk of developing diabetic nephropathy in Type I diabetic patients

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Abstract

Aims/hypothesis. Reports on a putative synergism between poor glycaemic control and carriage of the angiotensin II type 1 receptor (AGTR1) C¹¹⁶⁶-allele and risk of diabetic nephropathy have been conflicting. Therefore, we investigated the interaction between long-term glycaemic control and three polymorphisms in the genes coding for AGTR1 (A¹¹⁶⁶→C), angiotensin converting enzyme (ACE/ID) and angiotensinogen (M235T) on risk of developing diabetic nephropathy. Furthermore, we investigated the relation between a random measurement and long-term measurements of haemoglobin A_{1c} (HbA_{1c}).

Methods. We studied Caucasian patients with Type I (insulin-dependent) diabetes mellitus and nephropathy (120 men 74 women, age 41.1 ± 9.6 years, diabetes duration 28 ± 8 years) and long-standing Type I diabetic patients with persistent normoalbuminuria (112 men 69 women, age 42.5 ± 10.0 years, diabetes duration 27 ± 9 years). Genotyping was PCR-based and metabolic control estimated from all measurements of HbA_{1c} done in each patient [average (range) $n = 31$ (6–74)]. The median observation time (range) was 13.5 (2–14) years.

Results. Type I diabetic patients with a history of poor glycaemic control (HbA_{1c} above the median, 8.7%) had an increased risk of diabetic nephropathy compared with patients with a better metabolic control, OR (95% CI): 9.2 (5.8–14.7). The magnitude of this risk was similar in carriers and non-carriers of the mutations. The risk of nephropathy in patients with HbA_{1c} above compared with below the median in carriers of the mutant C¹¹⁶⁶-allele, D-allele, or T235-allele were 7.6 (95% CI: 3.9–14.8), 10.4 (6.0–17.8) and 9.8 (5.4–17.9), respectively. A significant correlation ($r = 0.74$, $p < 0.001$) existed between a random and long-term measurements of HbA_{1c} with a small mean difference (limits of agreement) [0.2 (–1.8 to 2.1) %] between the two estimates.

Conclusion/interpretation. Poor metabolic control is a major risk factor for diabetic nephropathy in Caucasian Type I diabetic patients. This risk was similar in carriers and non-carriers of the mutant alleles of the AGTR1(A¹¹⁶⁶→C), ACE/ID and angiotensinogen-M235T polymorphisms. The HbA_{1c} value measured at random reflects rather closely average long-term HbA_{1c} values. [Diabetologia (2000) 43: 794–799]

Keywords Renin-angiotensin system genes, glycaemic control, diabetic nephropathy, Type I diabetes.

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Abbreviations: RAS, Renin angiotensin system; AGTR1, angiotensin II type 1 receptor; ACE, angiotensin converting enzyme; ACE/ID, insertion/deletion polymorphism in the angiotensin converting enzyme gene; UAE, urinary albumin excretion rate.

The pathogenesis of diabetic nephropathy is multifactorial with contributions from haemodynamic, metabolic, growth and genetic factors [1]. In the search for genes underlying predisposition to diabetic nephropathy, the genes encoding components of the renin-angiotensin system (RAS) have attracted special attention. Several groups have investigated the relations between the genes encoding the angiotensin II type 1 receptor (AGTR1), angiotensin converting

enzyme (ACE) and angiotensinogen [2–9]. Regardless of the inconsistent results it is, however, unlikely that any of these loci has an effect large enough to account for the observed familial clustering of diabetic nephropathy in patients with Type I (insulin-dependent) diabetes mellitus [10–12].

Because hyperglycaemia is the best described and probably also the most important, contributor to the development of diabetic nephropathy [13, 14], studies on interaction between this risk factor and putative genetic risk factors are of major interest. Such an interaction would confound the interpretation of genetic studies and more importantly the existence of a synergistic interaction would identify carriers of mutant alleles as primary targets for metabolic prevention programmes of diabetic nephropathy.

The first report of such an interaction between poor glycaemic control and carriage of the AGTR1 C¹¹⁶⁶-allele on risk of diabetic nephropathy found an odds ratio of 12.1 in carriers and only 1.4 in non-carriers of the mutant allele [4]. A subsequent study did not, however, confirm this finding [15]. Short-term observation of glycated haemoglobin (HbA_{1c}) was applied in both studies. Whether such observations reflect glycaemic control over decades is still to be evaluated. The possible interaction between long-term poor metabolic control and the genes encoding components of the renin angiotensin system has not been explained.

Therefore, the aim of our study was to evaluate the interaction between long-term glycaemic control, assessed with HbA_{1c} and three polymorphisms in the genes coding for the AGTR1 (A¹¹⁶⁶→C), ACE (ACE/ID), and angiotensinogen (M235T) on the risk of developing diabetic nephropathy in Caucasian Type I diabetic patients. Furthermore, we investigated the relation between a random measurement and long-term measurements of HbA_{1c}.

Subjects and methods

Subjects. For the study of genetic risk factors of diabetic nephropathy [16, 17], we investigated three polymorphisms in the renin angiotensin system genes in 194 cases with Type I diabetes and diabetic nephropathy, diagnosed clinically based on the following criteria: persistent albuminuria greater than 300 mg/24 h in at least two out of three consecutive 24-h urine collections, presence of retinopathy and no clinical or laboratory evidence of other kidney or renal tract disease. A normo-albuminuric control group, matched individually to cases for sex, age and duration of diabetes, consisted of 181 Type I diabetic patients with previously persistent urinary albumin excretion rate (UAE) below 30 mg/24 h. A follow-up study of urinary albumin excretion rate in 1999 or at the time of death was carried out. Data were available for 93% of the cohort and showed that only 12 patients (7%) had progressed to microalbuminuria [median (range): 62 mg/24 h (35–242)], whereas none had progressed to macroalbuminuria.

In addition, all nephropathic Type I diabetic patients diagnosed between 1993 and 1997 were invited to participate in a

study of long-term glycaemic control. Thus, for this study the group of patients with diabetic nephropathy was enlarged by 137 subjects since the initial case-control study [16, 17].

The study was approved by the local ethics committee and all patients gave informed consent.

Methods. Glycaemic control was estimated from all measurements of HbA_{1c} done in each patient since referral to our clinic after 1983, when this analysis was implemented. The average number of measurements of HbA_{1c} was 31, range: 6–74. The mean of average yearly values was calculated for each patient. Median observation time was 13.5 (range 2–14) years. The method used for measurement of HbA_{1c} has changed over time: from 1983 to 1988, HPLC ion exchange [18] and isoelectric focusing [19] were used, these methods have a normal range of 4.1–6.1% and 4.1–6.4%. Thereafter HbA_{1c} was measured with HPLC (Bio-Rad Diamat, Richmond, Calif., USA) with a normal range of 4.1–6.4%. The correlation between this method and the two previous methods were $r = 0.983$ ($n = 194$) and $r = 0.931$ ($n = 119$), respectively. Finally, from 1994 a HPLC-based method was used (Bio-Rad Variant, Richmond, Calif., USA). The normal range remained unchanged (4.1–6.4%) and the coefficient of correlation between the latter and present method was $r = 0.993$ ($n = 161$).

Urinary excretion of albumin was determined from 24-h urine collections by enzyme immunoassay [20]. Serum creatinine was measured by a kinetic Jaffé method [21]. Lymphocytes were isolated from peripheral blood and DNA was prepared by standard techniques. The presence of the AGTR1 (A¹¹⁶⁶→C), ACE/ID and M235T polymorphism were determined by PCR and PCR-RFLP as described previously [2, 9, 16].

Statistical analysis. Values are given as means (SD) except for non-normally distributed variables, which are given as medians (range). Comparisons between groups of normal and log-normal distributed variables were done with an unpaired Student's t test. Frequencies are given as percentage with 95% confidence intervals. Odds ratios for presence of nephropathy was calculated for HbA_{1c} above compared with below the median value in carriers and non-carriers. The distribution of genotypes and alleles were compared with a chi-squared test. A logistic regression analysis was done with sex, age and duration of diabetes as covariates and presence of nephropathy as outcome variable. The independent variables were either genotype or carriers of the C-allele, D-allele, or T-allele, HbA_{1c} and their interaction. In addition, we analysed a model with HbA_{1c}, the three carrier-states, and the interaction of the three carrier states with each other. For comparison of a random compared with long-term measurements of HbA_{1c}, the two estimates are plotted as are the difference between methods against the mean [22]. Results are given as a correlation and the mean difference with limits of agreement. A p value (two-tailed) less than 0.05 was considered statistically significant. All calculations were made with commercially available programs (SPSS, SPSS, Chicago, Ill., USA) and R [23].

Results

The AGTR1 (A¹¹⁶⁶→C), ACE/ID and M235T polymorphisms were all determined in 375 patients. At the time of investigation, the group of patients with nephropathy (120 men 74 women, age $41.1 \pm$

Table 1. Risk of nephropathy associated with increased HbA_{1c} among carriers and non-carriers of C¹¹⁶⁶ – allele, D-allele, and T235-allele

	AGTR1 A ¹¹⁶⁶ → C polymorphism			
	C-allele carriers		C-allele non-carriers	
	Nephropathy	Normoalbuminuria	Nephropathy	Normoalbuminuria
Below median HbA _{1c} (8.7 %)	30	71	19	66
Above median HbA _{1c} (8.7 %)	61	19	84	25
Odds ratio (95 % CI)	7.6 (3.9–14.8)		11.7 (5.9–23.0)	
	ACE/ID polymorphism			
	D-allele carriers		D-allele non-carriers	
	Nephropathy	Normoalbuminuria	Nephropathy	Normoalbuminuria
Below median HbA _{1c} (8.7 %)	40	108	9	29
Above median HbA _{1c} (8.7 %)	115	30	30	14
Odds ratio (95 % CI)	10.4 (6.0–17.8)		6.9 (2.6–18.4)	
	Angiotensinogen M235T polymorphism			
	T-allele carriers		T-allele non-carriers	
	Nephropathy	Normoalbuminuria	Nephropathy	Normoalbuminuria
Below median HbA _{1c} (8.7 %)	29	86	20	51
Above median HbA _{1c} (8.7 %)	93	28	52	16
Odds ratio (95 % CI)	9.8 (5.4–17.9)		8.3 (3.9–17.8)	

9.6 years, diabetes duration 28 ± 8 years) and the normoalbuminuric group (112 men 69 women, age 42.5 ± 10.0 years, diabetes duration 27 ± 9 years) were well matched. The UAE was 801 (16–14545) mg/24 h in patients with nephropathy and 8 (1–30) mg/24 h in patients with normoalbuminuria. Some patients with previously persistent macroalbuminuria receiving antihypertensive medication had UAE below 300 mg/24 h at the time of investigation. Serum creatinine was increased in patients with nephropathy compared with the normoalbuminuric group [103 (54–684) vs 77 (40–116) $\mu\text{mol/l}$, $p < 0.001$]. The median HbA_{1c} based on all measurements during the observation period was 8.7 % for the study cohort ($n = 375$). The HbA_{1c} measurements were collected for a median period of 13.5 (2–14) years.

As described previously [2, 9, 16], there were no differences in AGTR1 (A¹¹⁶⁶→C), ACE/ID or M235T genotype or allele distribution between Type I diabetic patients with diabetic nephropathy and patients with long-standing Type I diabetes and persistent normoalbuminuria. Among control subjects, but not in cases, the distribution of ACE/ID genotypes differed significantly from the Hardy-Weinberg equilibrium, $p = 0.017$. The distributions of the M235T and AGTR1 (A¹¹⁶⁶→C) polymorphisms did not, however, deviate in either group.

A logistic regression analysis with sex, age and diabetes duration as covariates showed no significant associations between presence of nephropathy and the AGTR1 (A¹¹⁶⁶→C) [adjusted odds ratio (95 % CI) for C-allele carriers: 1.2 (0.7–2.1), $p = 0.44$], the ACE/ID [D-allele carriers: 1.4 (0.8–2.7), $p = 0.26$] or the M235T [T-allele carriers: 0.9 (0.5–1.6), $p = 0.75$]

polymorphism. Whereas the odds ratio associated with a difference of 1 % in average long-term value of HbA_{1c} was 5.0 (3.5–7.2) in this model, $p < 0.001$. Analysis of the interaction between HbA_{1c} and the investigated polymorphisms showed a change in OR of between 0.7 and 1.3 from carriers compared with non-carriers of the mutant alleles, all p values greater than 0.3. No gene-gene interactions were shown either.

When data was stratified according to carriage/non-carriage of the C¹¹⁶⁶-allele, D-allele and T235-allele and whether subjects had HbA_{1c} values above or below the median (8.7 %) (Table 1) Type I diabetic patients with a history of poor glycaemic control (HbA_{1c} > 8.7 %) had an increased risk of diabetic nephropathy compared with patients with a better metabolic control, OR (95 % CI): 9.2 (5.8–14.7). The magnitude of this risk was similar in carriers and non-carriers of the mutations (Table 1).

In addition, in patients with a history of HbA_{1c} greater than 8.7 %, carriers of the mutant alleles had no increased risk of developing diabetic nephropathy compared with non-carriers, OR: 1.0 (0.5–1.9), 1.8 (0.8–3.8) and 1.0 (0.5–2.1) for the AGTR1 (A¹¹⁶⁶→C), ACE/ID and M235T polymorphisms, respectively ($\chi^2 < 2.35$, 1 df; $p > 0.1$).

Exclusion of the 12 patients in the control group who subsequently progressed to microalbuminuria did not substantially alter results.

A significant correlation ($r = 0.74$, $p < 0.001$) existed between a random and long-term measurements of HbA_{1c} (Fig. 1). The Bland-Altman plot (Fig. 2) showed an average difference between random and long-term HbA_{1c} of 0.2 % with limits of agreement

Fig. 1. The relation between a random measurement of haemoglobin A_{1c} and the average of long-term measurements of haemoglobin A_{1c} . Type I diabetic patients with diabetic nephropathy (■) and persistent normoalbuminuria (□), $r = 0.74$, $p < 0.001$

Fig. 2. The difference between a random measurement and long-term measurements of haemoglobin A_{1c} as a function of the mean of random and long-term measurements. Type I diabetic patients with diabetic nephropathy (■) and persistent normoalbuminuria (□). Mean difference (limits of agreement) 0.2 (−1.8 to 2.1) %

−1.8 to 2.1 %. This is to a large extent attributable to a systematic difference, where random measures are higher for large values of HbA_{1c} and vice versa (regression to the mean).

Univariate linear regression analysis showed that glycaemic control remained unchanged over the whole observation period in 70 % of patients with nephropathy and had changed statistically signifi-

cantly over time in the remaining 30 % with an increasing HbA_{1c} in 59 of 100 patients. In the normoalbuminuric group the corresponding figures were 68 %, 32 % and 47 of 61 patients, respectively. Figure 3 shows the values of HbA_{1c} over time in the two groups.

In a subgroup of 226 patients with diabetic nephropathy, data on glycaemic control was available from the periods before and after onset of persistent proteinuria. Mean HbA_{1c} was 9.5 (1.4) % before the patients developed nephropathy and decreased thereafter to 9.3 (1.2) %, $p = 0.02$.

Discussion

Type I diabetic patients with long-standing poor glycaemic control had a greatly increased risk of diabetic nephropathy compared with patients with a better metabolic control. No synergistic effect between long-term poor glycaemic control and the mutant alleles of the AGTR1 ($A^{1166} \rightarrow C$), ACE/ID or angiotensinogen-M235T polymorphisms was found. Nor was a gene-gene interaction shown.

Random and long-term measurements of HbA_{1c} were closely correlated and the mean difference between the two values trivial.

A deviation from the Hardy-Weinberg equilibrium was observed for the ACE/ID genotypes in the original normoalbuminuric group. We have, however, no indications of population admixture in the Danish diabetic patients attending our clinic and a selective drop-out due to increased mortality is unlikely to be manifest only in the normoalbuminuric group, considered to be otherwise at relatively low cardiovascular risk. Finally, none of the other polymorphic markers investigated in the renin [24], angiotensinogen [9], and the AT₁-receptor gene [2] differed from the Hardy-Weinberg equilibrium in control or diabetic subjects in this study. Thus, selection is considered unlikely and the possibility of a chance finding remains likely.

Originally, a synergistic effect was suggested of the AGTR1 ($A^{1166} \rightarrow C$) polymorphism and poor metabolic control on risk of nephropathy in Type I diabetes ($n = 140$) with an odds ratio of 12.1 in carriers and only 1.4 in non-carriers of the mutant allele [4]. In the same paper, a similar pattern of higher risk of microalbuminuria was found in a cross-sectional study, although not statistically significant. A subsequent study [15] could not support this finding in a cohort from Northern Ireland ($n = 186$). Our study investigated more than twice the number of subjects and found no interaction between long-term glycaemic control and the AGTR1 ($A^{1166} \rightarrow C$) polymorphism on the risk of developing diabetic nephropathy in Caucasian Type I diabetic patients.

Whereas ethnic differences are unlikely to account for the difference between studies, selection of pa-

Fig. 3. The haemoglobin A_{1c} values during the follow-up period. Type I diabetic patients with diabetic nephropathy (■) and persistent normoalbuminuria (□)

tients could be a potential contributor: in the first study [4] a substantial number of patients with microalbuminuria were included, whereas only Type I diabetic patients with overt diabetic nephropathy were enrolled in the two European studies ([15], present study).

Furthermore, the methods used for the estimation of glycaemic exposure varies between studies: in one study [4] an index of the frequency of increased blood glucose concentration recorded at the Joslin clinic during the first 12 years of diabetes was used. The index is the percentage of the total number of blood glucose concentrations (in mmol/l) that exceeded: 10.0 fasting; 13.3 less than 1.5 h after eating; 12.2 1.5 to 2.4 h after eating; 11.1 2.5 to 3.4 h after eating; and 10.0 3.5 h or more after eating. In other studies, measurements of HbA_{1c} during the preceding 2 [4] or at least 1 year [15] was applied. In the present study a more accurate [25] estimate of long-term glycaemic control was calculated from all measurements of HbA_{1c} done at our clinic and thus an average of more than 30 measurements over 13 to 14 years was included in each patient.

Poor glycaemic control has been recognised as a major determinant of increased urinary albumin excretion rate in Type I diabetes in several studies [13, 14]. Consistent with that, HbA_{1c} was increased even before the development of persistent proteinuria in the present study. In addition, we found the risk of nephropathy to be more than seven times increased in patients with a HbA_{1c} above the median value, irrespective of allele carrier status. Similarly, in a previous study [4] the odds of nephropathy associated with an index of hyperglycaemia above the median was increased in carriers of the C¹¹⁶⁶-allele, whereas non-

carriers seemed to be protected against the effect of poor metabolic control. On the contrary, another study [15] found no increased risk of nephropathy in patients with poor glycaemic control either in carriers or in non-carriers of the C¹¹⁶⁶-allele. This apparent low risk of nephropathy with poor glycaemic control can be, however, ascribed to the small difference in mean HbA_{1c} concentration between the group of patients with (10.6%) and without (10.2%) nephropathy.

With respect to the ACE/ID polymorphism we did not find the D-allele to be a risk factor for diabetic nephropathy either before or after adjustment for long-term glycaemic control as suggested from a previous study of 63 micro-macroalbuminuric Type I diabetic patients [26]. Nor could we confirm a previous finding [27] of a gene-gene interaction between the ACE/ID and the M235T polymorphisms in our data.

Because the normoalbuminuric control subjects in our study have been less exposed to hyperglycaemia than diabetic subjects, an effect of a candidate gene able to modulate renal risk might have been masked. The finding of no interaction between any of the investigated polymorphisms and long-term glycaemic control in the present analyses does not, however, support this hypothesis.

In the majority of Type I diabetic patients the determination of HbA_{1c} on a single occasion offers a valid estimate of the average glycaemic exposure during the preceding decade. In accordance with a previous study [26] we found that within the usual therapeutic range (~ HbA_{1c} 7–11%) estimates of long-term glycaemic control are slightly lower than a single recent determination of HbA_{1c}. The mean difference is, however, small (0.2%) and without clinical relevance in the study of diabetic groups. For individual diagnostic and therapeutic purposes, valuable additional information can still be obtained from historical data of HbA_{1c}.

Poor metabolic control is a major risk factor for diabetic nephropathy in Caucasian Type I diabetic pa-

tients. This risk was similar in carriers and non-carriers of the mutant alleles of the AGTR1 (A¹¹⁶⁶→C), ACE/ID and angiotensinogen-M235T polymorphisms. A HbA_{1c} value measured at random reflects rather closely average long-term HbA_{1c} values.

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