

# Influence of Insertion/Deletion Polymorphism in the ACE-I Gene on the Progression of Diabetic Glomerulopathy in Type 1 Diabetic Patients With Microalbuminuria

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**OBJECTIVE** — To investigate the influence of the insertion/deletion polymorphism of the ACE gene on the progression of early diabetic glomerulopathy in patients with and without antihypertensive treatment (AHT).

**RESEARCH DESIGN AND METHODS** — There were 30 microalbuminuric patients with >5 years of type 1 diabetes who had renal biopsies taken at baseline and after 26–48 months of follow-up. Of the 30 patients, 13 (4 with II genotype and 9 with ID and DD genotypes) were randomized to AHT (enalapril or metoprolol) during the study. The ACE genotype was determined by a polymerase chain reaction. Glomerular structural changes were measured by stereological methods.

**RESULTS** — Of the patients, 8 had the II genotype, 19 had ID genotype, and 3 had DD genotype. During the study, basement membrane thickness, matrix star volume, and the overall diabetic glomerulopathy index were increased in patients with ID and DD genotypes only ( $P < 0.001$ ,  $P = 0.01$ ,  $P < 0.001$ , respectively). Among those with ID and DD genotypes, progression of basement membrane thickening and diabetic glomerulopathy index were increased in those without AHT, as compared with the antihypertensive treated patients ( $P < 0.001$ ,  $P = 0.02$ , respectively). In multivariate analysis, the ACE genotype had an independent influence on the progression of basement membrane thickening ( $P = 0.01$ ), when AHT ( $P < 0.001$ ) and the mean HbA<sub>1c</sub> during the study ( $P < 0.001$ ) were also taken into account. ACE genotype tended to be independently associated with the diabetic glomerulopathy index ( $P = 0.05$ ).

**CONCLUSIONS** — Microalbuminuric type 1 diabetic patients carrying the D-allele have an increased progression of diabetic glomerulopathy. Presence of this allele and no AHT seems to enhance this process. Larger studies are needed to confirm the clinical significance of our findings.

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Abbreviations: AER, albumin excretion rate; AHT, antihypertensive treatment; BMT, basement membrane thickness; BP, blood pressure; CVD, cardiovascular disease; DGP, diabetic glomerulopathy; I/D, insertion/deletion; PCR, polymerase chain reaction; s-ACE, s-angiotensin converting enzyme; Vv(mat/glom), matrix volume fraction per glomerulus; Vv(mes/glom), mesangial volume fraction per glomerulus.

A table elsewhere in this issue shows conventional and Système International (SI) units and conversion factors for many substances.

Sustained hyperglycemia is necessary for the development of diabetic nephropathy, but does not alone account for the occurrence of this disease (1). Diabetic nephropathy clusters in families (2), which can imply a hereditary trait for this complication. This is further supported by the fact that the incidence of diabetic nephropathy peaks during the second decade of type 1 diabetes and declines thereafter (i.e., when genetically susceptible individuals are supposed to be already affected).

We have recently found that familial hypertension and familial cardiovascular disease (CVD), independently of each other, are overrepresented among adolescents with type 1 diabetes and microalbuminuria (3). This may indicate that different genetic predispositions for these conditions are associated with increased risk for diabetic renal disease.

The insertion/deletion (I/D) polymorphism of the ACE gene may be involved in the pathogenesis of CVD (4,5). Plasma levels of ACE are strongly determined by the I/D genotype, with the lowest levels found in patients with the II genotype and the highest levels in those with the DD genotype. ACE levels in plasma are elevated in patients with type 1 diabetes, particularly in micro- and macroalbuminuric subjects (6). In addition, the inhibition of the renin-angiotensin system, which has a profound influence on renal hemodynamics and blood pressure (BP) regulation, retards the progression of microalbuminuria and the rate of decline in the glomerular filtration rate after the onset of diabetic nephropathy (7,8). Thus, the ACE gene has been a natural candidate in association analyses in clinical case-control studies regarding incipient or overt diabetic nephropathy. Results from such studies have been diverging (6,9–11), but a recent meta-analysis shows an association between nephropathy and the D-allele (12). It is still unknown, though, whether this allele may be involved in initiation and/or progression of the dis-

Table 1—Clinical data at baseline in patients with the II genotype and the ID and DD genotypes

	II genotype	ID and DD genotypes
n	8	22
Sex (M/F)	3/5	14/8
Age (years)	20.6 ± 5.4	19.2 ± 1.7
Duration of diabetes (years)	11.5 ± 3.2	11.2 ± 2.8
Insulin treatment		
Continuous subcutaneous insulin infusion	2	6
Conventional insulin treatment	6	16
AER (median [range]) (µg/min)	29 (18–160)	31 (15–194)
Antihypertensive treatment		
None	4	13
Beta-blocker	0	6
ACEI	4	3
S-ACE (U/ml)	21.4 ± 4.2	25.6 ± 6.4*
Mean HbA <sub>1c</sub> (%)	9.0 ± 1.3	9.0 ± 1.8
BP (mmHg)		
Mean systolic	122 ± 12	126 ± 9
Mean diastolic	77 ± 8	79 ± 7

Data are n, means ± SD, or medians (range). Mean HbA<sub>1c</sub>, systolic BP, and diastolic BP denote mean values during study. \*P = 0.05.

ease. Furthermore, the use of microalbuminuria or end-stage renal failure as clinical end points may suffer from a misclassification of disease or survival bias. Therefore, in the present longitudinal study of young microalbuminuric type 1 diabetic patients with and without antihypertensive treatment (AHT), we wanted to investigate the influence of the I/D polymorphism of the ACE gene on the progression of diabetic renal structural lesions that underlie the development of clinical nephropathy.

## RESEARCH DESIGN AND METHODS

### Subjects

We included 30 patients >15 years old with >5 years of type 1 diabetes and with persistent microalbuminuria (i.e., >15 µg/min in at least two of three consecutive overnight urine samples) in this study. Patients were recruited in two different departments of pediatrics in Stockholm (n = 13) and Oslo (n = 17). They were enrolled in two different studies regarding the effect of AHT (13) and intensive insulin treatment (14) on the progression of diabetic glomerulopathy. The two patient groups had similar age (18.8 ± 2.1 vs. 20.1 ± 3.5 years), duration of diabetes (11.1 ± 3.4 vs. 11.4 ± 2.5 years), and degree of microalbuminuria (31 [19–160] vs. 30 [15–194] µg/min). All participants had fundus photography performed before the

study. Simplex retinopathy (one or more microaneurysms or hemorrhages) was present in 10 of the Stockholm patients and 14 of the Oslo patients. None had proliferative changes and no patient was prescribed a low-protein diet.

### Procedure

All subjects had two renal biopsies performed with 26- to 48-month intervals. Immediately after the baseline renal biopsy, the Stockholm patients were randomly allocated to treatment with either an ACE inhibitor (enalapril 20 mg/day, n = 7) or a beta-blocker (metoprolol 100 mg/day, n = 6). The Oslo patients were randomized to either conventional insulin treatment (two or multiple subcutaneous injections daily, n = 9) or continuous insulin infusion (n = 8) (14). None of the Oslo patients received AHT.

The HbA<sub>1c</sub>, albumin excretion rate, and BP were measured every second (14) or every third month (13).

The HbA<sub>1c</sub> was analyzed by high-performance liquid chromatography methods (Auto-A; Kyoto-Diaichi, Kagaku, Kobe, Japan [13] and Bio-Rad, Richmond, CA [14]). Reference levels were 4.0–6.0% and 4.3–6.1%, respectively. The AER was analyzed by an immunoturbidimetric method (15), from timed overnight urine samples from all patients.

Systolic and diastolic BPs were measured by an automatic device (DINAMAP;

Critikon, Johnson-Johnson, Tampa, FL) (13) or by a conventional mercury sphygmomanometer (14).

The baseline renal biopsy s-angiotensin converting enzyme (s-ACE) levels were analyzed in all patients in the same laboratory, and thereafter every 6 months in the Stockholm patients (13) using a radioimmunoassay method (Buhlman Laboratories AG, Basel, Switzerland). The reference level was 18–55 U/ml.

### DNA analysis

At baseline, a blood sample was collected and later used for the analysis of I/D polymorphism of the ACE gene. DNA was isolated from frozen EDTA-full blood samples using the Puregene kit (Gentra Systems, Minneapolis, MN). The isolated DNA samples were subjected to genotyping of the ACE-polymorphism by polymerase chain reaction (PCR), as previously described (16). PCR was performed with 1 µg DNA using sense oligo 5'-CTGGAGACCACTCCATCCTTTCT-3' and anti-sense oligo 5'-GATGTCGCCATCACATTTCGTACAT-3' as primers, in a 20-µl solution containing 3 mmol/l MgCl<sub>2</sub>, 50 mmol/l KCl, 10 mmol/l Tris-HCl, pH 8.4, 0.1 mg/ml gelatin, 0.5 mmol/l each of dNTP (Gibco-BRL) and 2U Taq polymerase (Gibco-BRL, Rockville, MD). Amplification was performed with 32 cycles at 94°C for 45 s, 55°C for 45 s, and 45 s at 73°C. The PCR products were separated by gel electrophoresis in a 2% agarose gel containing ethidium bromide. The primers were situated outside of an I/D area in intron 16 of the ACE gene, hereby giving rise to PCR products of either 490 bp (insertion allele) or 190 bp (deletion allele). All individuals were genotyped twice with identical results.

### Morphometric methods

Ultrasound guided transcutaneous renal biopsies were taken using an automatic device with an 18-gauge needle (Precision Cut TM AB; Becton Dickinson, Franklin Lakes, NJ and Biopsy; Bard, Covington, GA, respectively). The tissue was immediately fixed in 2% glutaraldehyde in a modified Tyrodes buffer and delivered to the Electron Microscopy Laboratory in Aarhus, Denmark. There it was sectioned into smaller blocks, dehydrated, and embedded into Epon (TAAB Laboratories, Aldermaston, Berkshire, U.K.) (13) or into Vestopal (Serva Feinbiochemica, Heidelberg, Germany) (14). The same morphometric methods were used for all patients

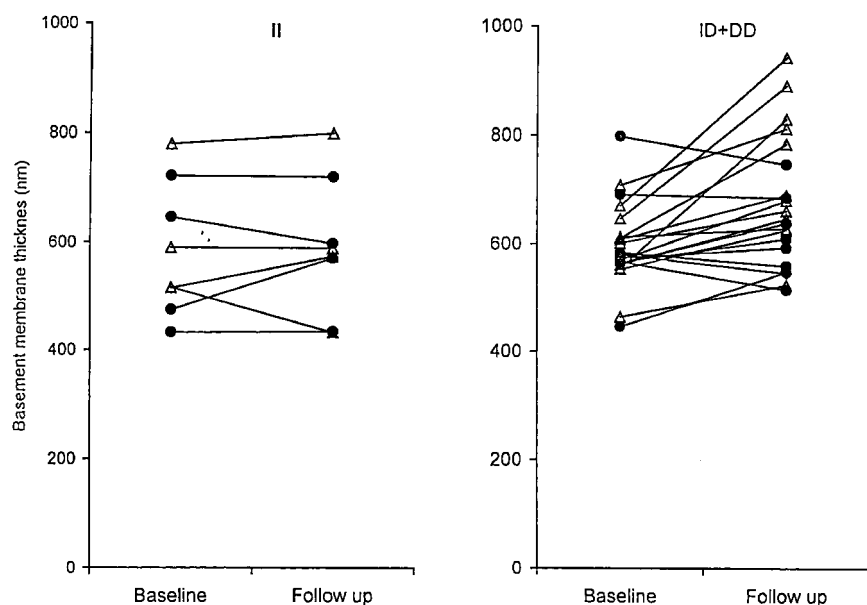


Figure 1—BMT at baseline and follow-up in 8 patients with the II genotype and 22 patients with the ID and DD genotypes (control mean 350.51 nm). ●, Patients with AHT; △, patients without AHT.

and all of the samples were analyzed at the same laboratory.

Quantitative structural data were obtained by electron microscopy using strictly systematic independent sampling of glomeruli. Serial 1- $\mu\text{m}$ -thick sections were cut, picked up on slides, and stained with toluidine blue. The first section of the block, having full tissue, was the baseline section. Thin sections for electron microscopy were cut in new-appearing glomeruli in the block, i.e., sampling independent of glomerular size and morphology. The levels for thin sectioning were predetermined to be at 50  $\mu\text{m}$  and multiples thereof from the baseline section, i.e., with a random independent position within the glomeruli. In each of three new-appearing glomeruli the first three levels were used for electron microscopy.

Quantitation by electron microscopy. Two levels of magnification were used. At low magnification ( $\times 2,300$ ), the entire glomerular profile was photographed to produce photomontages, i.e., three complete cross-sections were obtained in each of three glomeruli. On the montages, the mesangial volume fraction ( $V_v$  [mes/glom]) was estimated with an 8:1 grid and a point distance between fine points of  $\sim 8 \mu\text{m}$ . At high magnification ( $\times 9,800$ ), a subsample of the area ( $\sim 24\%$ ) was photographed in the largest of the three profiles in each glomerulus. On the micrographs, the basement membrane thickness (BMT) was estimated by orthogonal intercepts (17), and the matrix volume fraction within the

mesangium volume fraction ( $V_v$  [mat/mes]) was estimated with a 2:1 grid. Finally, matrix star volume was measured by classifying point-sampled intercepts in a predetermined direction (18).

#### Statistical methods

Due to a small number of patients with the DD genotype, subjects with the ID and DD

genotypes were treated together in the statistical analyses. Comparisons between patients with II versus ID and DD genotypes were performed by unpaired Student's *t* test. Before comparisons,  $\Delta$ -values of morphometric measures were adjusted for follow-up time and calculated per 24 months, assuming a linear change. When comparing baseline and follow-up data, paired Student's *t* test was used. To evaluate the relative influence of ACE genotype on morphometric changes, multiple regression analyses were performed using ACE genotype as a dichotomized variable, i.e., II/ID and DD (0/1). AHT was dichotomized as yes/no (0/1). Data are presented as means  $\pm$  SD, except for AER, in which case median (range) is given.

**RESULTS**—Eight patients had the II genotype, 19 had the ID genotype, and 3 had the DD genotype. The allele frequencies were 0.58 for I and 0.42 for D. No statistically significant deviation was found from the expected genotype distribution according to the Hardy-Weinberg equilibrium.

Follow-up time was similar between genotype groups. Age, sex, and duration of diabetes did not differ between patients with II genotype and ID and DD genotypes. AHT (either the ACE inhibitor enalapril 20 mg/day or the beta-blocker metoprolol 100 mg/day) was used during follow-up in 50% of patients with the II genotype and 41% of

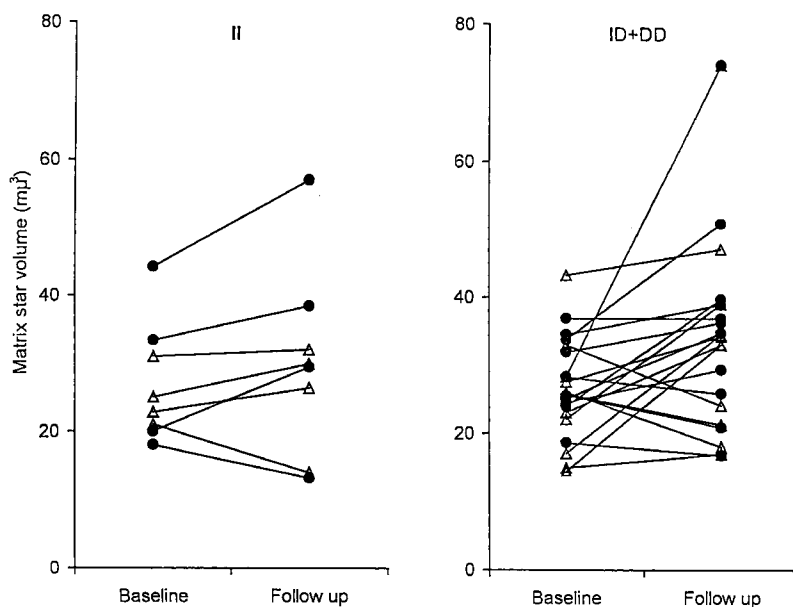


Figure 2—Matrix star volume at baseline and follow-up in 8 patients with the II genotype and 22 patients with the ID and DD genotypes (control mean  $14.2.8 \text{ m}^3$ ). ●, Patients with AHT; △, patients without AHT.

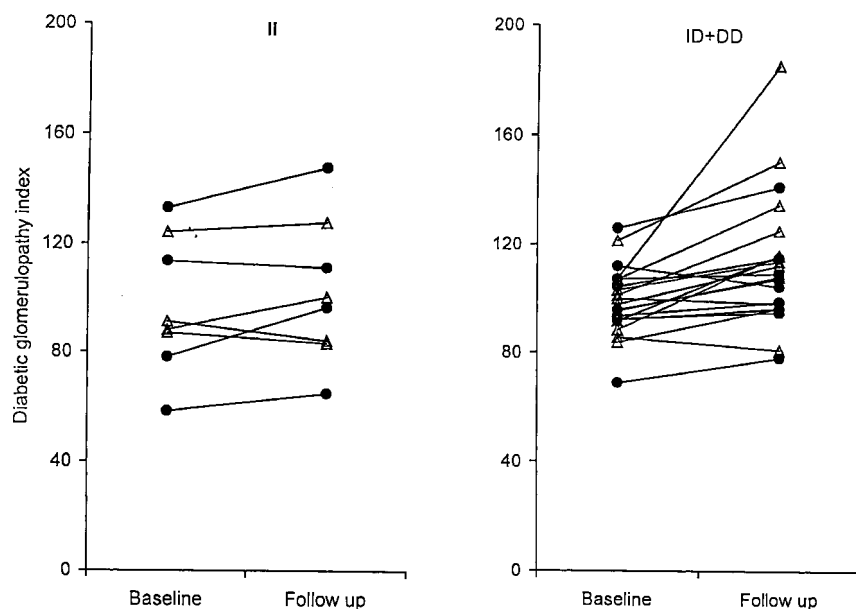


Figure 3—Overall index DGP at baseline and follow-up in 8 patients with the II genotype and 22 patients with the ID and DD genotypes (control mean 58.28). ●, Patients with AHT; △, patients without AHT.

patients with the ID and DD genotypes. At baseline (before treatment) AER was similar between groups, whereas s-ACE was higher in the ID and DD group (Table 1). At final follow-up median, AER was 11 (6–134)  $\mu\text{g}/\text{min}$  in the II genotype group and 14 (4–187)  $\mu\text{g}/\text{min}$  in the ID and DD genotype group ( $P = 0.32$ ).

Mean  $\text{HbA}_{1c}$  and mean systolic and diastolic BPs, calculated from measurements every 6 months, were similar in patients with the II and ID and DD genotypes (Table 1).

#### Morphological results

BMT, matrix star volume, and overall DGP index, i.e., index DGP ( $\text{BMT}/10 + \text{Vv}(\text{mat}/\text{glom})\% + \text{matrix star volume}$ ) were similar at baseline but increased during follow-up in patients with ID and DD genotypes only (Figs. 1–3).  $\text{Vv}(\text{mes}/\text{glom})$  and  $\text{Vv}(\text{mat}/\text{glom})$  did not increase in any group (Table 2). The increase in BMT over 24 months was significantly higher in ID and DD genotypes as compared with II genotype ( $P = 0.01$ ), and so was the increase in index DGP over 24 months ( $P = 0.03$ ). The increase in matrix star volume over 24 months tended to be higher in patients with ID and DD genotypes ( $P = 0.09$ ).

In patients with ID and DD genotypes, an increased progression in basement membrane thickening ( $P < 0.001$ ) and in

index DGP ( $P = 0.02$ ) was seen in patients without AHT, as compared with those with AHT (Figs. 1 and 3). Thus, to study the independent relationship between the ACE genotype and  $\Delta\text{BMT}$  over 24 months and  $\Delta\text{index DGP}$  over 24 months, multiple regression analyses were performed, also including AHT and mean  $\text{HbA}_{1c}$  during the study as independent variables. It was found that mean  $\text{HbA}_{1c}$  ( $P < 0.001$ ), no AHT ( $P < 0.001$ ), and ID and DD genotype ( $P = 0.01$ ) had independent significant influences on  $\Delta\text{BMT}$  over 24 months. When using  $\Delta\text{DGP}$ -index as the dependent variable, only mean  $\text{HbA}_{1c}$  had a significant influence on this morphometric measure ( $P = 0.01$ ). ACE genotype and AHT tended to have an independent influence on the change in DGP-index ( $P = 0.05$  and  $0.07$ , respectively).

Table 2—Baseline and follow-up structural data in patients with the II genotype and the ID and DD genotypes

	II genotype		ID and DD genotypes	
	Baseline	Follow-up	Baseline	Follow-up
BMT (nm)	584 $\pm$ 123	590 $\pm$ 126	600 $\pm$ 77	686 $\pm$ 131*
Vv(mes/glom) (%)	21.6 $\pm$ 5.4	21.9 $\pm$ 4.4	20.7 $\pm$ 2.3	21.4 $\pm$ 3.5
Vv(mat/glom) (%)	11.9 $\pm$ 2.7	13.0 $\pm$ 3.4	12.1 $\pm$ 2.6	12.5 $\pm$ 2.3
Matrix star volume ( $\mu\text{m}^3$ )	26.9 $\pm$ 8.8	30.0 $\pm$ 13.9	26.6 $\pm$ 7.1	35.4 $\pm$ 15.6†
Index DGP	96.7 $\pm$ 25.0	102.0 $\pm$ 26.6	99.2 $\pm$ 11.6	116.7 $\pm$ 8.1*

Data are means  $\pm$  SD. \* $P < 0.001$ ; † $P = 0.01$ .

**CONCLUSIONS**— We have previously demonstrated that improved metabolic control, as well as AHT, may retard the development of glomerular structural changes in microalbuminuric type 1 diabetic patients (13,14). To evaluate the possible additional role of genetic susceptibility, we have now extended the study of these patients by investigating the relative influence of the I/D ACE-gene polymorphism on the progression of diabetic glomerulopathy. Despite the small number of patients, we find that those heterozygous or homozygous for the D-allele show an increased progression in BMT and overall diabetic glomerulopathy index. To our knowledge, such data have not been presented earlier. However, our results are in agreement with several (6,9) but not all (10,11) clinical studies, using either progression of microalbuminuria or decline in glomerular filtration rate as end points. In addition, in the meta-analysis by Fujisawa et al. (12) that included 4,773 diabetic patients, it was suggested that the D-allele has a major impact on susceptibility for nephropathy.

Our data also indicate that the influence of the D-allele is independent of AHT, at least on the progression of BMT. More importantly, young microalbuminuric patients hetero- or homozygous for the D-allele, but without AHT, seem to have a more marked progression of diabetic glomerulopathy during a 2-year period than those with AHT. It is noteworthy that the structural changes at baseline were in the same range in the two subgroups. This observation is compatible with the idea that the ACE-polymorphism may not be associated with the initialization of nephropathy. Tarnow et al. (19) have previously suggested that the ACE-inhibitor captopril may have a less renal-protective effect in macroalbuminuric type 1 diabetic patients with the D-allele. In that study, the degree of albuminuria was unchanged despite capto-

pril treatment in patients with the DD genotype. In contrast, the results from the present study indicate that patients carrying the D-allele and receiving any AHT (either beta-blocker or ACE-inhibitor) acquire a retarded progression of BMT and overall diabetic glomerulopathy, as compared with those without AHT. However, based on this study, any conclusive answer regarding the effect of such treatment on patients with the DD genotype alone cannot be drawn due to the small number of these patients. In addition, our patients were in an early phase of diabetic renal disease with only minimal mesangial expansion that did not progress over the study period. It may be speculated that the stage of nephropathy is decisive for the response to AHT in patients with different ACE genotypes. Moreover, the number of patients treated with ACE-inhibitor in our study is low.

A poor long-term metabolic control is a well-known risk factor for diabetic nephropathy (1). As expected, in our morphological study, mean HbA<sub>1c</sub> during follow-up was a significant determinant for the progression of BMT and overall DGP. These results are compatible with those of Barnas et al. (9) who found that the 10-year mean HbA<sub>1c</sub> and DD genotype were independent risk factors for the prevalence of diabetic nephropathy.

The increased progression of diabetic glomerulopathy, seen in our patients with ID and DD genotypes, may be ascribed to either the I/D ACE-gene polymorphism itself or to adjacent disease mutations, e.g., 1Dde polymorphism (20). Clearly, several other putative genetic markers not investigated in this study may also be involved in the progression of glomerulopathy (20). Furthermore, this is a small study combining two patient groups and data must be regarded with some caution. On the other hand, the precise morphological parameters may justify results of rather small series. A main finding of interest is that young type 1 diabetic patients with microalbuminuria carrying the D-allele and not receiving AHT are at higher risk for progression of diabetic glomerulopathy. Our results need to be confirmed by other large-scale studies, also taking other putative confounders into account. This must happen before an analysis of ACE-gene polymorphism in young normotensive type 1 diabetic patients, at the very early stage of microalbuminuria, can be accepted as a simple tool for deciding who will benefit most from AHT.

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