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Improvement of blood glucose control in IDDM patients retards the progression of morphological changes in early diabetic nephropathy

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Summary We investigated in a randomized, prospective study the influence of improved blood glucose control during 2–3 years in young insulin-dependent diabetic (IDDM) patients with microalbuminuria, which is indicative of early nephropathy. Patients were randomized either to intensive treatment by continuous subcutaneous insulin infusion (CSII) ($n = 9$) or CT ($n = 9$). Kidney biopsies were taken at baseline and after 26–34 months. End points were structural changes in the glomeruli. Sensitive, quantitative, morphometric methods were used. The blood glucose control improved significantly ($p = 0.01$) during the study in the CSII-group as glycated haemoglobin (HbA_{1c}) fell from 10.1% ([95% CI] 8.9–11.3) to 8.6% (7.9–9.2), but not in the CT-group, 10.1% (8.3–11.9) vs 9.7% (8.7–10.8). Mean HbA_{1c} during the study period was significantly lower in the CSII-group than in the CT-group, 8.7% (8.1–9.3) vs 9.9% (8.5–11.3), $p = 0.04$. Basement membrane thickness (BMT) increased in both groups, most (CT vs CSII, $p = 0.03$) in the CT-group: 140 nm (50–230) vs CSII: 56 nm (27–86). In the CT-group only an increase was seen in matrix/mesangial volume fraction ($p = 0.006$) and matrix star volume ($p = 0.04$). Furthermore, a positive correlation between mean HbA_{1c} during the study and change from baseline in BMT ($r = 0.70$, $p = 0.001$) and matrix/glomerular volume fraction ($r = 0.33, p = 0.09$, NS) was demonstrated. Albumin excretion rate correlated significantly to BMT and most of the matrix parameters. The present study shows that during a period of only 2.5 years, a close relationship between the level of mean blood glucose and progression of glomerular morphological changes in early diabetic nephropathy can be demonstrated. [Diabetologia (1994) 37: 483–490]

Key words Diabetic glomerulopathy, microalbuminuria, basement membrane thickness, mesangial expansion, mesangial matrix, stereology, hyperglycaemia.

Even though hyperglycaemia is a prerequisite for the development of diabetic nephropathy, the impact of long-term hyperglycaemia on the progression of early diabetic nephropathy is not well understood. In 1978 Pirart [1] showed in a study comprising both IDDM and NIDDM patients, that the risk of development of serious complications including nephropathy, is associated with more severe hyperglycaemia. In cross-sectional [2–6] and long-term retrospective studies [7, 8] an association between the level of glycated haemoglobin and microalbuminuria, which is an early sign of diabetic nephropathy [9, 10], has been found. Improved blood glucose control obtained in prospective randomized studies has retarded the progression of AER [11] and the risk of developing clinical nephropathy [12, 13]. However, it is not known if reducing mean blood glucose affects the progression of morphological changes at a very early stage of diabetic nephropathy. Therefore we tested in a randomized, prospec-

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Abbreviations: IDDM, insulin-dependent diabetes mellitus; CSII, continuous subcutaneous insulin infusion; CT, conventional treatment (2, 3 or multiple injections daily); BMT, basement membrane thickness; AER, urinary albumin excretion rate; CI, confidence interval.

tive intervention study the hypothesis that the progression rate of characteristic morphological changes in early diabetic nephropathy is related to mean blood glucose level.

Subjects, materials and methods

Subjects

The initial study comprised 371 IDDM patients between 10–30 years of age with diabetes duration of more than 5 years who were screened for persistent microalbuminuria. The patients were non-proteinuric as demonstrated by a negative Albustix (Boehringer Mannheim GmbH Mannheim, Germany) and came from five different out-patient clinics (282 subjects from Aker University Hospital, Paediatric and Medical Departments, and 98 from local Paediatric Departments). Persistent microalbuminuria was defined as an AER between 15–200 μg/min in at least two out of three overnight urine samples taken during 1 year. The lower AER-limit of 15 μg/min, rather than the conventional 20 μg/min, was chosen since we used overnight urine samples. The AER is reduced by 25% during the night compared to 24-h samples [14]. We invited the 45 patients (12%), who satisfied this criterion, to take part in a prospective 2-year trial, of whom 33 patients agreed. This paper describes the 18 patients who were above 18 years of age in whom renal biopsy could be performed. Each of the patients received thorough information and gave their written consent. The protocol was approved by the Regional Ethics Committee. No serious complication of the kidney procedure was observed. One patient had macroscopic haematuria for 1 day without any other signs.

The patients were then randomly assigned to CSII by a portable pump or CT (multiple injections or two–three injections per day). Before the study 27 of the patients were on multiple injections, three, on two daily injections and three, on three daily injections. The randomization was carried out by a computer program in the following priority in order to make the best possible distribution of basic characteristics: mean 1-year pre-study HbA_{1c}, duration of diabetes, median 1-year pre-study AER, age and sex. The statistician doing this had no knowledge of the patients identification or mode of treatment.

Three patients were excluded from the controlled, prospective study. The reasons were incidence of alcohol abuse, atopic eczema, and problems with the fixation of the subcutaneous needle, respectively, making pump treatment inconvenient.

The characteristics of the 18 patients who completed the 2-year study, are shown in Table 1. The patients did not have proliferative retinopathy and all but one patient had blood pressure less than 140/90 mm Hg. This patient was initially hypertensive (150/98 mm Hg), but the blood pressure declined to 145/85 mm Hg in the course of 6 months without antihypertensive treatment.

The patients were followed regularly by the same investigator (HJB) at the out-patient clinic at 2-month intervals. Renal function tests and biopsies were performed at entry and after 26–34 months also in the three patients who were excluded from the randomized study.

Table 1. Individual clinical data at entry from 18 IDDM subjects on CSII or CT

Subject no	Age (years)	Duration (years)	AER (μg/min)	HbA_{1c} (%)	Blood pressure (mm Hg)	
					Systolic	Diastolic
CSII:						
1	18	10	15	10.3	110	85
2	21	9	59	9.1	115	78
3	18	12	19	13.7	110	78
4	23	18	16	8.8	137	67
5	18	7	25	10.1	134	78
6	29	15	40	8.4	138	78
7	18	11	30	10.7	118	80
8	19	10	18	9.8	114	69
9	19	12	131	10.3	134	90
Mean	20.3	11.6	39	10.1	123	78
95% CI	17–23	9–14	11–68	8.9–11.3	114–133	73–84
CT:						
10	19	10	33	12.5	145	88
11	19	12	30	8.6	135	90
12	18	12	194	13.8	120	80
13	20	8	40	10.7	125	85
14	18	13	50	12.5	118	76
15	18	12	32	9.1	118	78
16	19	10	37	7.8	150	98
17	19	12	18	8.0	120	75
18	29	12	27	7.9	115	85
Mean	19.9	11.2	51	10.1	127	84
95% CI	17–23	10–12	10–93	8.3–11.9	117–137	78–90

[a] Mean values of 2–4 measurement in the year preceding the study

Methods

AER was measured in at least two timed overnight urine samples in the year preceding the study, and then at 2-month intervals. The albumin concentration was measured by immunoturbidimetry in samples kept at 4°C from 1 to 3 days. The inter-assay coefficient of variation was 4.7% in the range of 10–50 mg/l. The urine samples were negative for leucocytes, nitrite, albumin and ketones as demonstrated by Nephur-Test Leuco (Boehringer-Mannheim GmbH).

HbA_{1c} was analysed with an HPLC method ("Diamat" analyzer; Biorad, Richmond, Calif., USA). Normal range was 4.3–6.1%, with an inter-assay coefficient of variation of 3%.

Blood pressure was measured by conventional mercury sphygmomanometer with patients sitting after a 10-min rest, and diastolic pressure was recorded at the disappearance of the Korotkoff's V sound.

Glomerular filtration rate was measured by inulin-clearance (Inulin; Laevosan, Linz, Austria) after oral water loading. High concentrations of interfering glucose were removed by glucose oxidase. Glomerular filtration rate was standardized to a body surface area of 1.73 m^2.

The renal charge selectivity index was measured as the ratio between the urinary clearances of two endogenous molecules, IgG and IgG_4 as previously described [15]. These molecules have the same size and configuration, but IgG_4 is more anionic than total-IgG, the isoelectric points = 5.5–6.0 vs 5.8–7.3, respectively [16].

Renal biopsies

In the diabetic patients ultrasound guided kidney biopsies were taken with an 18 gauge needle integrated automatic gun-biopsy system (Biopty, Bard Inc, Covington, Ga., USA). The tissue was

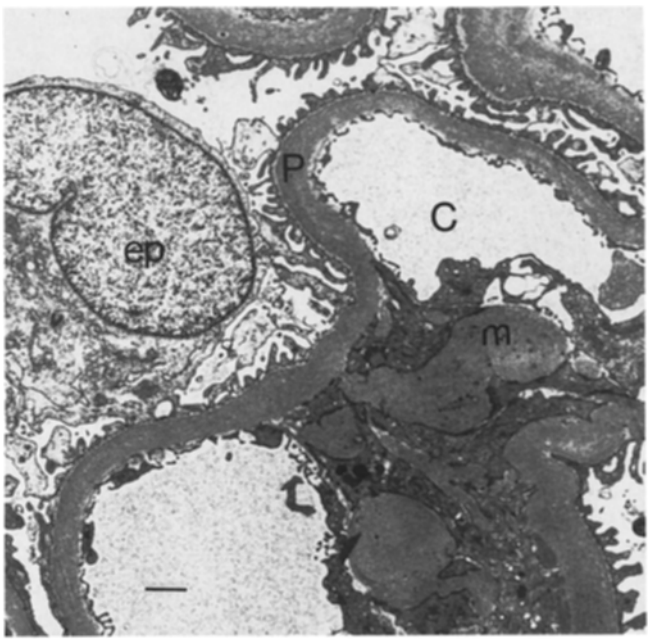

Fig. 1. Section of glomerular tuft. The separation between the peripheral basement membrane (p) and the mesangial matrix (m) is indicated. Epithelial cell: ep; capillary space: c. The bar equals 1 μm

immediately immersed into the fixative, a 2% glutaraldehyde solution in modified Tyrode buffer and mailed in the fixative to the laboratory in Århus. Embedding into Vestopal was done after 3–10 days storage in the fixative. The investigator (RØ) was not aware of the group designation of the subjects.

Measurement of structural parameters

The methods for obtaining quantitative structural parameters in the glomerulus have been described recently [10]. Three glomeruli from each biopsy were sampled independently of size and structure, except for one biopsy in which only two glomeruli were obtained. The structures in question are peripheral basement membrane, the mesangial region and the mesangial matrix.

Photomontages of the entire glomerular profile were made at a magnification of 2350 × at three levels in each glomerulus. The set of three sections, separated by 60 μm, had a random position along the glomerular diameter, and was used for the estimate of mesangial volume fraction and surface densities of the peripheral BM, mesangial-urinary and the mesangial-capillary surfaces. A systematic subsample of the largest of the three sections was photographed at 9900 × magnification and was used for measurements of BM thickness and mesangial matrix.

Thickness of the peripheral basement membrane (BMT): the delineation between peripheral BM and the mesangial matrix was defined as illustrated in Figure 1. Measuring points were sampled independently of the BM appearance with a line grid, measurements taken at intersections between grid lines and the endothelial-BM interface. The 'true BM thickness' was estimated [17], classifying only at the sampling places, where the distinctness of the epithelial cell membrane showed that the section was perpendicular to the BM surface.

Mesangial volume fraction, V_V(mes/glom), was estimated by point counting (an 8: 1-grid) using the circumscribed polygon as reference space [10]. *Mesangial matrix* was estimated as volume fraction of mesangium, V_V(mat/mes), using a 2: 1-grid. The product of the two volume fractions gives *matrix as fraction of glomerular space*, V_V (matrix/glom). *Matrix star volume* was obtained by measuring linear intercepts in the matrix, from random points within matrix, in a direction with a 3-dimensional uniform orientation distribution [18]. The point-sampled intercepts (l_o) were classified with a ruler. The matrix star-volume, $V^* = (\pi/3) \cdot (l_0^3)$. The star volume is proportional with confluence and/or convexity of the matrix. Increasing star volume implies a high degree of separation of mesangial cells and their segments. *Matrix-thickness*, matrix-T, was estimated as the ratio of V_V(matrix/tuft)/S_V(mesangial-urinary surface/tuft).

Statistical analysis

Comparisons between the two subsets of patients, on CSII or CT, respectively, were done by unpaired, Student's *t*-test, changes over time by paired Student's *t*-test, and linear correlations were tested by least squares regression to the mean (Number Cruncher Statistical System, Kaysville, Utah, USA). The a priori hypotheses were that CSII would lead to improved blood glucose control and would lessen the expected progression of morphological lesions. Therefore, one-sided tests were used. As for the relationship between AER level and morphological lesions, the a priori hypothesis was that higher AER is associated with more advanced lesions. Therefore correlations between AER and morphology were tested one-sided. AER-values were logarithmically transformed before statistical analysis and then antiloged. Results are presented as mean with 95% CI. Statistical significance was accepted with *p*-values less than 5%.

Since the idea in this study was not to test the efficacy of insulin pumps vs multiple injections, but rather to test the effect of improved metabolic control, all patients were considered together in correlation analyses of change in structural parameters vs HbA_{1c} during the study.

Results

The patients on intensified treatment (CSII) improved their HbA_{1c} from 10.1% (8.9–11.3%) at entry to 8.6% (7.9–9.2) at the end of the study ($p = 0.01$). No significant reduction, 10.1% (8.3–11.9%) vs 9.7% (8.7–10.8%), was observed in the CT-group. The mean HbA_{1c} (nine measurements per patient) during the study period, was lower in the CSII-group than in the CT-group, 8.7% (8.1–9.3) vs 9.9% (8.5–11.3), $p = 0.04$.

The development of the structural changes is presented in Table 2. The CSII-group showed a significant increase during the study only for BM thickness, whereas the CT-group increased in BMT, matrix/mesangial volume fraction, and matrix star volume. The increase in BMT (Fig. 2) and matrix/mesangial volume fraction was significantly higher in the CT- than in the CSII-group, whereas the difference in matrix/glomerular volume fraction ($p = 0.051$) and matrix star volume ($p = 0.11$) did not reach statistical significance. Neither group showed any change in mesangial/glomerular volume fraction.

Table 2. Structural parameters in 18 IDDM patients on CSII or CT. Results at baseline (B) and follow-up (F) after 26–34 months

Subject no	Basement membrane thickness (nm)		Matrix/mesangial volume fraction		Matrix/glomerular volume fraction		Mesangial/glomerular volume fraction		Matrix star volume (μm^3)		Matrix thickness (μm)	
	B	F	B	F	B	F	B	F	B	F	B	F
CSII												
1	553	626	0.61	0.61	0.12	0.12	0.19	0.19	33	24	3.3	3.1
2	602	662	0.66	0.57	0.15	0.13	0.22	0.23	23	33	2.7	3.7
3	709	813	0.59	0.65	0.14	0.14	0.24	0.22	22	39	3.6	4.2
4	613	629	0.53	0.57	0.11	0.13	0.21	0.23	23	33	2.1	3.8
5	570	681	0.56	0.57	0.12	0.11	0.21	0.20	15	17	3.1	2.7
6	780	800	0.52	0.63	0.15	0.16	0.29	0.25	31	32	3.7	4.9
7	590	589	0.59	0.60	0.11	0.11	0.19	0.19	21	14	3.2	3.2
8	465	524	0.60	0.54	0.13	0.11	0.22	0.20	26	18	2.3	2.4
9	742	806	0.66	0.55	0.16	0.14	0.24	0.26	38	43	3.8	3.7
Mean	625	681	0.59	0.59	0.13	0.13	0.22	0.21	26	28	3.1	3.5
95 % CI	548–702	601–760	0.55–0.63	0.56–0.62	0.12–0.15	0.12–0.14	0.20–0.25	0.20–0.24	20–31	20–36	2.6–3.6	2.9–4.1
B vs F	$p = 0.001$		NS		NS		NS		NS		NS	
CT												
10	372	524	0.62	0.68	0.14	0.14	0.23	0.21	25	28	2.8	2.7
11	562	647	0.59	0.64	0.12	0.10	0.21	0.16	35	39	3.6	2.7
12	671	942	0.58	0.65	0.12	0.17	0.21	0.26	28	74	3.5	5.9
13	647	890	0.54	0.68	0.14	0.14	0.25	0.21	43	47	3.0	3.9
14	555	830	0.50	0.54	0.11	0.12	0.21	0.22	26	21	4.6	3.1
15	611	785	0.63	0.65	0.13	0.12	0.20	0.19	28	34	2.6	2.9
16	608	691	0.58	0.63	0.10	0.13	0.18	0.20	17	35	2.7	3.3
17	516	433	0.55	0.57	0.13	0.14	0.23	0.24	23	26	2.4	2.5
18	515	574	0.61	0.59	0.12	0.13	0.19	0.22	25	30	3.7	2.9
Mean	562	702	0.57	0.63	0.12	0.13	0.21	0.21	28	37	3.2	3.3
95 % CI	493–631	569–835	0.54–0.61	0.59–0.66	0.11–0.13	0.12–0.15	0.20–0.23	0.19–0.23	22–33	25–49	2.7–3.8	2.5–4.1
B vs F	$p = 0.004$		$p = 0.006$		$p = 0.12$, NS		NS		$p = 0.04$		NS	
CSII vs. CT (Δ)	$p = 0.03$		$p = 0.04$		$p = 0.052$, NS		NS		NS		NS	

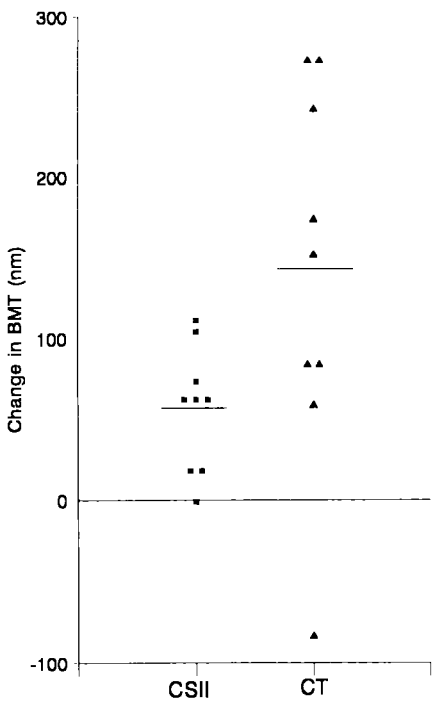

Fig. 2. Change in BMT during 26–34 months. The difference between the two groups of subjects, IDDM patients on CSII and CT respectively, is statistically significant ($p = 0.03$)

The correlation analysis between mean HbA_{1c} of the study period and the change (Δ) from baseline to follow-up in the structural parameters showed a strong association between mean HbA_{1c} and Δ-BMT ($r = 0.70$, $p = 0.001$, Fig. 3). Furthermore, a positive, but not significant, correlation was found between mean HbA_{1c} and Δ-matrix/glomerular volume fraction ($r = 0.33$, $p = 0.09$) and matrix/mesangial volume fraction ($r = 0.26$, $p = 0.15$).

The AER showed a very modest increase in both groups; in the CSII-group from 27 (10–75) µg/min to 34 (12–98) µg/min, and in the CT-group from 28 (12–67) µg/min to 32 (12–83) µg/min. The increase did not reach statistical significance in either group. Even though all of the patients had persistent microalbuminuria before the study, some had AER values less than 15 µg/min at baseline and occasionally during the study.

The correlation between AER and the respective structural parameters at the beginning of the study was: BMT ($r = 0.46$, $p = 0.03$), matrix/glomerular volume fraction ($r = 0.34$, $p = 0.08$, NS), matrix/mesangial volume fraction ($r = 0.31$, $p = 0.10$, NS), matrix star volume ($r = 0.12$, NS), matrix thickness ($r = 0.15$, NS), and mesangial/glomerular volume fraction ($r = 0.14$, NS). At the end of the study an even closer association

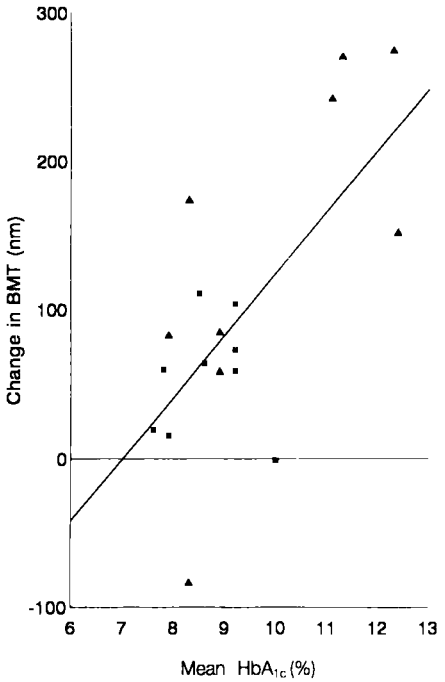

Fig. 3. Change in BMT vs mean HbA_{1c} during 26–34 months in 18 subjects with IDDM ($r = 0.70$, $p = 0.001$). ■ CSII, ▲ CT

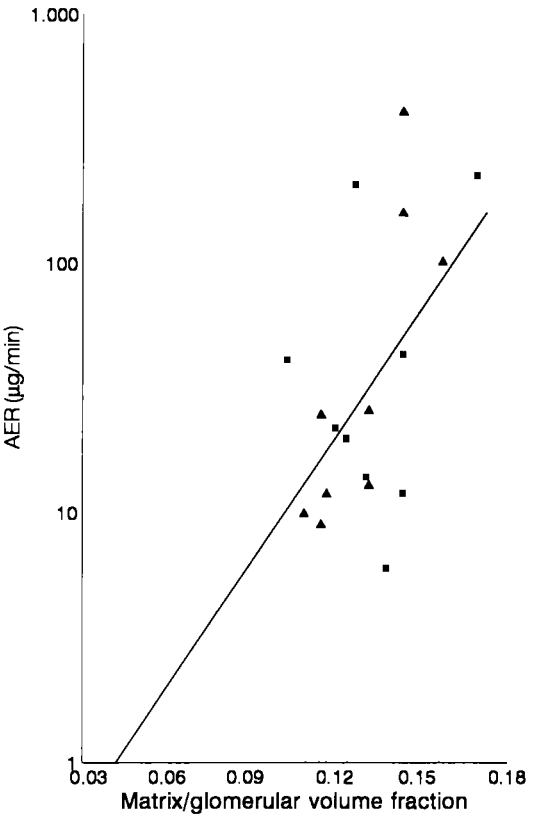

Fig. 4. AER in 18 subjects with IDDM vs matrix/glomerular volume fraction at the end of the study period ($r = 0.52$, $p = 0.01$). ■ CSII, ▲ CT

was observed: BM thickness ($r = 0.58$, $p = 0.006$), matrix/glomerular volume fraction (0.52, $p = 0.01$) [Fig. 4], matrix star volume ($r = 0.59$, $p = 0.005$), matrix thickness (0.58, $p = 0.006$), and mesangial/glomerular volume fraction ($r = 0.41$, $p = 0.04$). Further, the changes (Δ) in structural parameters during the study, ΔBMT ($r = 0.44$, $p = 0.03$), Δmatrix star volume ($r = 0.69$, $p = 0.001$), Δmesangial/glomerular volume fraction ($r = 0.52$, $p = 0.01$), Δmatrix/glomerular volume fraction ($r = 0.40$, $p = 0.05$, NS), and Δmatrix thickness ($r = 0.42$, $p = 0.04$) showed a positive correlation with mean AER the year preceding the study.

Blood pressure remained unchanged in both groups during the study period. Δ Systolic blood pressure was –2.2 mm Hg (–9.8 – 5.4) vs 1.3 mm Hg (–5.6 – 8.3), and Δ diastolic blood pressure 2.6 mm Hg (–4.0 – 9.1) vs 0.4 mm Hg (–5.0 – 5.4), in the CSII- and the CT-group respectively. No correlation was found between blood pressure and change in structural parameters.

The glomerular filtration rate showed no significant change in either of the two groups (data not shown). In the two groups combined glomerular filtration rate was at baseline 143 (130–156) $ml \cdot min^{-1} \cdot (1.73\ m^2)^{-2}$ and 150 (136–165) $ml \cdot min^{-1} \cdot (1.73\ m^2)^{-2}$ at follow-up and did not correlate with BMT.

The charge selectivity index, clearances of (IgG/IgG_4), was not correlated to BMT at the beginning of the study. However a negative correlation between the increase in basement membrane thickness and change in charge selectivity index was observed ($r = -0.73$, $p = 0.001$, Fig. 5).

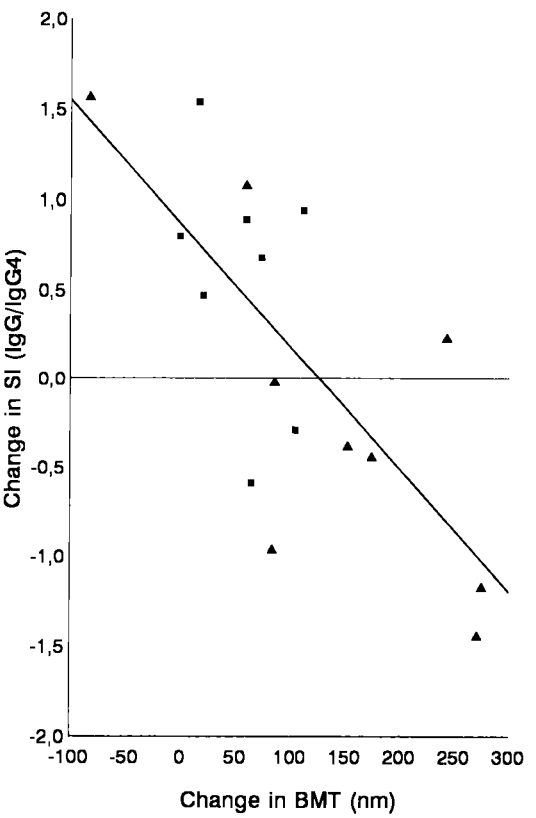

Fig. 5. Change in selectivity index (IgG/IgG_4) vs change in BMT during 26–34 months in 17 (in one of the 18 patients we did not obtain adequate urine and serum measurements of IgG and IgG_4 [11]) subjects with IDDM ($r = 0.73$, $p < 0.001$). ■ CSII, ▲ CT

Discussion

The present randomized, prospective study shows that in IDDM patients with microalbuminuria, an increase of BMT and matrix expansion takes place during a period of 2 to 3 years. The increment is significantly larger in the group randomized to CT than in those on intensive treatment by CSII.

In the total group of patients the increment of BM thickness and matrix/glomerular volume fraction during the study showed a significant positive correlation with mean HbA_{1c}. Hitherto, no prospective study has shown similar data in human native diabetic kidneys. Two previous reports dealt with the effect of pancreas transplantation on kidney allograft in diabetic patient [19, 20]. One showed that normalization of blood glucose was followed by less severe glomerulopathy, i.e. less increase in BMT and mesangial volume fraction [19], and the other study that the increment of the mesangial volume fraction, but not the BM-thickening, was halted after 12 years [20]. In the cross-sectional study of our series at baseline, we found an association between HbA_{1c} and BM thickness and matrix star volume, respectively [10]. As discussed [10] a high precision in the estimates was mandatory for the study. With the use of three levels in each glomerulus the variation in mesangial estimates was considerably reduced.

It is not known which constituents are responsible for the early thickening of BM and matrix expansion in diabetes. In the BM collagen IV, laminin and heparansulphate proteoglycan predominate while the mesangial matrix also contains collagen V, fibronectin, trombospondin, and chondroitin/dermatan sulphate proteoglycans [21]. Short-term experimental studies show that hyperglycaemia induces increased production of the aforementioned proteins, the main constituents of the extracellular material [22–26]. Furthermore, hyperglycaemia leads to accumulation of advanced glycated end-products of proteins. These glycated proteins do contribute to the formation of pathological tissue deposits [27].

In controlled, prospective, intervention studies with a limited number of patients, improved blood glucose control retards progression of AER [11] and reduces the risk of developing clinical nephropathy [12, 13]. The Diabetes Control and Complications Trial confirms these results in a large-scale study, even though the design of the study focused on retinopathy [28]. An association between the degree of AER and structural parameters has only been found when a "structural index", which included BMT and matrix volume fraction, was constructed [9, 29]. In the present study the results were very consistent: at the end of the study, AER showed a positive correlation with all of the structural parameters. The same pattern was found at entry, but not to the same extent. The patients with the highest AER levels at the beginning of the study, showed the most marked progression in structural parameters. These relationships were observed, irrespective of the well-known, large intra-individual variation of AER [30]. It should be noted that in the last part of the the study, 4 of the 21 patients had AER-values below 15 μg/min in repeated urine samples and thus by definition no longer had microalbuminuria [31].

The cause of hypertension in diabetic nephropathy and the relative importance of hypertension in the early phases of diabetic nephropathy, is still controversial [32]. In the study reported by Chavers et al. [33], the structural changes in microalbuminuric patients were most pronounced in those patients who also had either elevated blood pressure or reduced glomerular filtration rate, a category that is not represented in our series. We found no correlation between blood pressure and the changes in structure, but the blood pressure was within normal limits and the range was rather narrow. This may indicate that blood pressure at this early stage of nephropathy does not play a major role in the initiation of the early structural lesions.

The long-term prognosis in diabetic nephropathy is linked to loss of capillary surface in the advanced stages. Thickening of the peripheral BM, as well as expansion of mesangial regions, in particular the matrix, are integral elements in the pathological picture at this stage. Our studies have shown again that the thickening of peripheral BM and the mesangial matrix expansion seem to develop in parallel. We observed a striking correlation between the increment of BM thickness and loss of negative charge, as investigated by the selectivity index, clearances of (IgG/IgG_4), indicating that the thickened BM is likely to be qualitatively changed. Thus, loss of anionic charge may be an early event in the development of diabetic nephropathy, as suggested by Deckert et al. [16], and may be explained by decreased sulphatation of heparan sulphate proteoglycan in the glomerular basement membrane. This process is also hampered in the matrix and it is proposed that this abnormality contributes to the development of diabetic glomerulopathy [24].

We have shown that improved blood glucose control retards the development of structural changes in early diabetic nephropathy. This is found, even with a modest improvement in HbA_{1c} and without achieving optimal blood glucose control. An HbA_{1c}-level below 7.5–8.0% is probably sufficient to avoid progression of urinary albumin excretion [34]. Blood glucose values corresponding to this level can be achieved without an increased risk of serious hypoglycaemia [35, 36]. Furthermore, the relationship between AER and structural parameters, gives additional support to the clinical importance of detecting elevated albumin excretion in the early stage of diabetic nephropathy when intervention is still potentially beneficial.

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