Alterations in serum lipids and apolipoproteins in male Type 1 (insulin-dependent) diabetic patients with microalbuminuria

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Summary. The relationships between serum lipid, apolipoprotein levels and urinary albumin excretion were investigated in 20 male Type 1 (insulin-dependent) diabetic patients with microalbuminuria (overnight urinary albumin excretion between 10 and 200 µg/min), in 18 male Type 1 diabetic patients without microalbuminuria and in 18 male control subjects. In the microalbuminuric patients low density lipoprotein cholesterol was higher than in the control subjects ($p < 0.05$); the high density lipoprotein/low density lipoprotein cholesterol ratio was lower than in the normoalbuminuric diabetic patients ($p < 0.05$), and in the control subjects ($p < 0.01$); apolipoprotein B was higher than in the normoalbuminuric patients ($p < 0.05$); the apolipoprotein A₁/B ratio was lower than in the normoalbuminuric diabetic patients ($p < 0.05$). Serum triglyceride was higher in the microalbuminuric diabetic patients and in the control subjects than in the normoalbuminuric diabetic patients ($p < 0.05$, for both), but was not different be-

tween the microalbuminuric diabetic patients and the control subjects. No significant differences between the 3 groups were present with respect to serum cholesterol, high density lipoprotein cholesterol and apolipoprotein A₁. In the 2 combined Type 1 diabetic groups there were significant correlations between urinary albumin excretion and the high density lipoprotein/low density lipoprotein cholesterol ratio ($R = -0.40$, $p < 0.02$), apolipoprotein B ($R = 0.35$, $p < 0.05$) and the apolipoprotein A₁/B ratio ($R = -0.44$, $p < 0.01$). These results indicate microalbuminuria related differences in lipid and apolipoprotein levels in male Type 1 diabetic patients, which may contribute to an increased risk of cardiovascular disease.

Key words: Lipoproteins, apolipoproteins, microalbuminuria, incipient nephropathy, Type 1 (insulin-dependent) diabetes mellitus.

Cardiovascular disease is the major cause of premature death in Type 1 (insulin-dependent) diabetes mellitus [1–3]. Diabetic patients are also at risk from the cardiovascular risk factors such as smoking, hypertension and serum lipid levels. Epidemiological studies have shown that the excess risk for cardiovascular mortality is observed predominantly in those patients, who have developed diabetic nephropathy [4, 5]. Indeed, a higher prevalence of the risk factors of smoking and elevated blood-pressure appears to be present in patients with proteinuria [6–10]. Many reports have shown unaltered or increased levels of HDL cholesterol, normal LDL cholesterol levels and normal or low apolipoprotein B (apo B) levels in Type 1 diabetes, provided metabolic control is adequate and kidney function is well preserved [11–18]. In contrast, the presence of diabetic nephropathy and possibly also retinopathy is associated with lipoprotein abnormalities which harbour an increased risk for cardiovascular disease [9, 10, 19–22]. Diabetic nephropathy, hallmarked by persistent proteinuria, is preceded

by a phase of incipient nephropathy, which is characterised by microalbuminuria [23–25].

The present study was conducted to investigate possible alterations in lipid and apolipoprotein levels in Type 1 diabetic patients with early renal lesions as indicated by microalbuminuria.

Subjects and methods

All subjects consented to the procedure after explanation of the purpose of the study, which was approved by the local medical ethics committee. Three groups of subjects were investigated (Table 1).

- a) 20 Type 1 diabetic patients with microalbuminuria (urinary albumin excretion rate (Ualb.V) ranging from 10 to 200 µg/min): D Micro.
- b) 18 Type 1 diabetic patients with normal Ualb.V (less than 10 µg/min): D Normo.
- c) 18 control subjects (Ualb.V less than 10 µg/min): C.

The level of Ualb.V was determined in 3 consecutive overnight urine collections obtained within 3 months prior to the study. Three urine

Table 1. Clinical characteristics of the 3 groups of diabetic and non-diabetic subjects

	D Micro (n=20) ^a	D Normo (n=18) ^a	C (n=18) ^a
Age (years)	46 (21–60)	43 (22–69)	41 (21–63)
Duration of diabetes (years)	18 (6–40)	18 (2–41)	–
Ualb.V (µg/min)	29 (10–228)	3 (1–9) ^e	3 (1–6) ^e
Serum creatinine (µmol/l)	85 ± 14	82 ± 11 ^c	87 ± 6
Overnight creatinine clearance (ml · min ⁻¹ · 1.73 m ²⁻¹)	102 ± 24	109 ± 20	105 ± 20
Glomerular filtration rate (ml · min ⁻¹ · 1.73 m ²⁻¹)	128 ± 31	n.d.	n.d.
Body mass index (kg/m ²)	24.23 ± 2.33	24.00 ± 1.84	22.83 ± 2.07
Insulin dose (U · kg ⁻¹ · day ⁻¹)	0.78 ± 0.24	0.70 ± 0.18	–
Smokers (n)	11	7	4
HbA _{1c} (%)	8.23 ± 1.17 ^d	7.55 ± 1.24 ^d	5.46 ± 0.67
Fasting blood glucose (mmol/l)	12.44 ± 3.00 ^d	10.73 ± 4.22 ^d	4.50 ± 0.37
Serum albumin (g/l)	43.35 ± 2.37 ^c	43.61 ± 2.38 ^c	45.28 ± 2.40
Mean blood pressure (mm Hg)	95 ± 8	94 ± 8	95 ± 6
Retinopathy (O/B/P) ^b	4/10/6	9/8/1	–

^a D Micro: Type 1 (insulin-dependent) diabetic patients with microalbuminuria; D Normo: Type 1 diabetic patients without microalbuminuria; C: Control subjects

^b Retinopathy: O: absent; B: background; P: proliferative.

Results are given as mean ± SD or median (range) as indicated

^c denotes $p < 0.05$ from C; ^d denotes $p < 0.01$ from C; ^e denotes $p < 0.01$ from D Micro

collections were used for classifying patients because of the high coefficient of variation of Ualb.V (of about 45%) [26]. A level of 10 µg/min represents the upper normal limit of Ualb.V in our clinic. Levels persistently above 200 µg/min, corresponding with a urinary protein excretion above 500 mg/24 h, indicate diabetic nephropathy. Urinary tract infection was excluded by bacterial culture. Serum creatinine was below 120 µmol/l in all subjects. Between the 3 groups no differences in creatinine clearance determined in overnight urine collections could be demonstrated. The glomerular filtration rate, measured with ¹²⁵I-iothalamate [27] was determined only in D Micro and was above 85 ml · min⁻¹ · 1.73 m²⁻¹ in all tested subjects.

The Type 1 diabetic patients were recruited from the outpatient clinic and, by the use of multiple daily insulin injections, were in a metabolically stable condition. All the patients suffered from ketosis prone diabetes mellitus and were deemed insulin-dependent on clinical grounds. The control subjects were recruited from hospital personnel. No subject had arterial hypertension (systolic blood pressure > 160 mm Hg, diastolic blood pressure (Korotkoff phase 5) > 90 mm Hg). Only male subjects were recruited thus avoiding any effect of the menstrual cycle [28] or the use of oral contraceptives on lipid levels. None of the subjects used any medication other than insulin known to affect lipoprotein levels or kidney function. No subject suffered from thyroid or liver disease as determined by routine thyroid and liver function tests.

As is shown in Table 1, the 3 groups were closely matched for age and body mass index. The 2 Type 1 diabetic groups were matched for diabetes duration which was at least 2 years. The levels of Ualb.V, given in Table 1, are those from overnight collections obtained direct-

ly before blood sampling. In D Micro all values were above 10 µg/min, but one subject had Ualb.V above 200 µg/min (228 µg/min). There were no significant differences in metabolic control between D Micro and D Normo. On average metabolic control assessed by HbA_{1c} was adequate. The mean fasting blood glucose values were higher than normal. There was no significant difference in insulin dose between the 2 diabetic groups. Retinopathy tended to be more severe in D micro ($0.05 < p < 0.10$). In D Micro more smokers were present, but the prevalence of smoking was not significantly different between the 3 groups ($p > 0.20$). Three patients from D Micro and 1 patient from D Normo had suffered from a myocardial infarction (at least 6 months previous to the study). One patient from D Normo was suffering from ischaemic cerebrovascular disease. No one in C had cardiovascular disease.

Laboratory methods

Venous blood was drawn after a 12 h fast. The last administration of insulin was 12 h before blood sampling. Blood used for lipid and apolipoprotein determinations was allowed to clot. Serum was separated at 3000 rev/min for 12 min within 1–2 h. Samples were frozen at –20°C and assayed within 4 weeks. The assays were performed in duplicate in one run.

Lipid assays

Lipids were measured in serum and the HDL containing supernatant after precipitation of apo B containing lipoproteins with sodium phosphotungstate and MgCl₂ [29]. Cholesterol and triglyceride were measured enzymatically using automated methods [30, 31]. LDL cholesterol was calculated by the Friedewald formula [32]. Apolipoprotein A₁ (apo A₁) and B were determined by immunoturbidimetry on a Multistat I centrifugal analyser (Instrumentation Laboratories, IJsselstein, The Netherlands) using commercially available kits (Boehringer, Mannheim, FRG, cat. no. 726478 and 726494, respectively). Coefficient of variation 5.2 and 4.6%, respectively.

Other techniques

Urinary albumin was measured by a commercially available double antibody radioimmunoassay (Diagnostic Products Corporation, Apeldoorn, The Netherlands, cat. no. KHAD₂). Serum and urinary creatinine were measured on a SMAC II autoanalyser (Technicon Instruments Inc., Tarrytown, NY, USA). HbA_{1c} was determined by colorimetry [33]. Glucose was measured on a Yellow Springs glucose analyser (YSI Model 23A, Yellow Springs Inc., Yellow Springs, Ohio, USA).

Statistical analysis

Results are expressed as mean ± SD for normally distributed data and as median and range for non-parametrically distributed data. p values less than 0.05 were considered to be significant. Differences in prevalence of variables among groups were analysed by chi-square analysis [34]. Comparison of data among groups was performed by analysis of variance for parametrically and non-parametrically distributed data as appropriate. Adjustment for multiple comparisons was carried out using Duncan's method [35]. Spearman's rank correlation coefficients were used for the assessment of relationships between variables for the combined 2 groups of Type 1 diabetic patients [34]. Subsequently multiple stepwise linear regression analysis was carried out to evaluate the independence of relationships. In this analysis log₁₀ transformed Ualb.V values were used.

Results

The serum concentrations of lipids and apolipoproteins are given in Table 2. Between the 3 groups there were no significant differences in serum cholesterol levels. Serum triglyceride was slightly lower in D Normo ($p < 0.05$) than in D Micro and in C. No differences between D Micro and C were found. HDL cholesterol was not significantly different in the 3 groups. LDL cholesterol levels were higher in D Micro than in C ($p < 0.05$).

The HDL/LDL cholesterol ratio was lower in D Micro than in D Normo ($p < 0.05$) and in C ($p < 0.01$). No differences in apo A₁ levels could be demonstrated. Apo B was higher in D Micro than in D Normo ($p < 0.05$). The difference between D Micro and C was close to statistical significance ($0.05 < p < 0.10$). The apo A₁/apo B ratio was lower in D Micro than in D Normo ($p < 0.05$). The serum concentrations of lipids and apolipoproteins were also compared in the non-smokers and smokers from the 3 groups separately. Among the non-smokers the HDL/LDL cholesterol ratio was lower in D Micro (0.32 ± 0.10) than in C (0.39 ± 0.11 , $p < 0.05$). Among the smokers the apo A₁/apo B ratio was lower in D Micro (1.28 ± 0.38) than in D Normo (1.59 ± 0.28 , $p < 0.05$). In D Micro LDL cholesterol and apo B tended to be higher both in the non-smokers and smokers, but the differences were not significant in these small groups of subjects.

The correlation coefficients between Ualb.V and the various lipid parameters in the combined 2 groups of Type 1 diabetic patients are given in Table 3. The Ualb.V values used are those from one overnight collection obtained directly before lipid determinations. There was a positive correlation between Ualb.V and apo B ($p < 0.05$) and there were negative correlations between Ualb.V and the HDL/LDL cholesterol ratio ($p < 0.02$) and the apo A₁/apo B ratio ($p < 0.01$). Multiple linear regression analysis was performed to determine the independence of these relationships. It could be demonstrated that the relationships of Ualb.V and apo B, HDL/LDL cholesterol ratio, apo A₁/apo B ratio were independent. These relationships were not attributable to other clinical parameters including the number of cigarettes smoked daily, age, diabetes duration, body mass index, insulin dose, HbA_{1c}, blood glucose and degree of retinopathy. No significant correlations could be demonstrated of Ualb.V and serum cholesterol, serum triglyceride, LDL cholesterol and apo A₁. The inverse correlation with HDL cholesterol was close to statistical significance ($0.05 < p < 0.10$). Furthermore, there was a positive correlation between Ualb.V and the degree of retinopathy ($R 0.32$, $p < 0.05$). The independence of this relationship was confirmed by multiple linear regression analysis. There was also a negative correlation between the degree of retinopathy and the apo A₁/apo B ratio ($R -0.34$, $p < 0.05$). This relationship, however,

Table 2. Serum concentrations of lipids and apolipoproteins in the 3 groups

	D Micro ^a	D Normo ^a	C ^a
Cholesterol (mmol/l)	6.01 ± 1.19	5.51 ± 0.79	5.40 ± 0.96
Triglyceride (mmol/l)	0.92 (0.35–2.80)	0.79 (0.47–2.56) ^{b, d}	1.07 (0.51–2.03)
HDL cholesterol (mmol/l)	1.15 ± 0.34	1.27 ± 0.19	1.27 ± 0.19
LDL cholesterol (mmol/l)	4.28 ± 1.06^b	3.86 ± 0.69	3.63 ± 0.82
HDL/LDL cholesterol	0.29 ± 0.12^c	0.34 ± 0.06^d	0.37 ± 0.11
Apolipoprotein A ₁ (g/l)	1.28 ± 0.24	1.24 ± 0.15	1.28 ± 0.15
Apolipoprotein B (g/l)	0.97 ± 0.28	0.79 ± 0.17^d	0.83 ± 0.17
Apolipoprotein A ₁ /B	1.41 ± 0.47	1.70 ± 0.44^d	1.57 ± 0.37

^a D Micro, D Normo and C: for explanation see Table 1

^b denotes $p < 0.05$ from C; ^c denotes $p < 0.01$ from C; ^d denotes $p < 0.05$ from D Micro

Table 3. Correlation coefficients between urinary albumin excretion rate and serum lipid parameter in the 2 combined groups of 38 Type 1 (insulin-dependent) diabetic patients

	Urinary albumin excretion rate
Cholesterol	0.13
HDL cholesterol	–0.28
LDL cholesterol	0.12
HDL/LDL cholesterol	–0.40 ^b
Triglyceride	0.24
Apolipoprotein A ₁	–0.12
Apolipoprotein B	0.35 ^a
Apolipoprotein A ₁ /B	–0.44 ^c

^a denotes $p < 0.05$; ^b denotes $p < 0.02$; ^c denotes $p < 0.01$
Spearman rank correlation coefficients are given

disappeared in multiple linear regression analysis and could be attributed to the correlation between Ualb.V and the apo A₁/apo B ratio.

Discussion

The present cross sectional study demonstrates alterations in lipid and apolipoprotein profiles in Type 1 diabetes with renal lesions as early as incipient nephropathy. In association with microalbuminuria higher LDL cholesterol and apo B concentrations and lower HDL/LDL cholesterol and apo A₁/apo B ratios were observed. These differences in lipid and apolipoprotein levels are related to an increased risk of cardiovascular disease [36, 37]. Lower HDL/LDL cholesterol and apo A₁/apo B ratios in the microalbuminuric patients were also demonstrable when the non-smokers and smokers were analysed separately. This indicates that the alterations in lipids and apolipoproteins in the microalbuminuric patients were not merely attributable

to smoking which is known to be related to microvascular diabetic complications and unfavourable lipid profiles [6, 7, 38]. Furthermore, in the combined 2 Type 1 diabetic groups independent relationships between urinary albumin excretion and apolipoprotein B, HDL/LDL cholesterol, apo A₁/apo B were found. No significant differences in serum cholesterol could be measured. Serum triglyceride was slightly higher in the microalbuminuric and the control group than in the normo-albuminuric group. There was no relationship between urinary albumin excretion and serum triglyceride.

Previous studies have shown lipoprotein abnormalities in diabetic nephropathy [10, 19, 21, 22]. In incipient nephropathy no significant differences in HDL, LDL, and VLDL cholesterol and an increase in apo B have been reported [21, 22]. In that study no correlation between urinary albumin excretion and apo B could be demonstrated [21]. While the present study was under consideration, two reports have appeared also showing alterations in lipids and apolipoproteins in microalbuminuria [39, 40]. In our study the overnight urinary albumin excretion rate, considered to be abnormal (above 10 µg/min), was low. Lipoprotein abnormalities may, therefore, be present even when the urinary albumin excretion rate is only slightly increased. A relationship between retinopathy and lipid levels has been observed [19, 20]. In the present study a negative correlation between the degree of retinopathy and apo A₁/apo B ratio was found, but this relationship disappeared upon correction for urinary albumin excretion.

The mechanisms behind the alterations in lipids and apolipoprotein levels found in microalbuminuria and proteinuria remain to be elucidated. In the nephrotic syndrome an increased hepatic lipoprotein synthesis as well as an impaired catabolism has been documented [41, 42]. Leakage in the urine of HDL has been reported in nephrotic rats and non-diabetic nephrotic man [42-44] and may contribute to a low HDL/LDL cholesterol ratio. Serum levels of lipoproteins are, at least in part, genetically determined among other factors through apo B and apo E polymorphism and inherited differences in LDL subclasses [45-47]. These possibilities have not yet been investigated.

When studies from non-diabetic populations, showing a 2% decrease in cardiovascular death per % decrease in serum cholesterol [48] are extrapolated to the diabetic population, it is clear that differences in lipid levels of the magnitude presently demonstrated for microalbuminuria do not explain the highly increased cardiovascular mortality in patients who have developed diabetic nephropathy. Such an assumption may not be valid however. In vitro studies have shown that extravasated lipoproteins may be trapped in the arterial wall to a greater extent in diabetes due to increased cross linking with advanced glycosylation end products on extra cellular matrix proteins [49]. Macrophages accumulate more cholesterylester when exposed

to glycosylated LDL in vitro [50]. These mechanisms would lead to a decrease of cholesterylester outflux from the arterial wall and would promote atherogenesis. In diabetes, complicated by microalbuminuria, outflux from plasma of proteins, especially albumin, is increased [51]. A concomitant increase in the outflux of other macromolecules might thus promote atherogenesis particularly in patients with renal lesions [50, 52]. It seems possible, therefore, that the cardiovascular risk associated with a small increase in lipid levels is higher for diabetic patients with renal lesions than for diabetic patients without renal lesions and for normal subjects. It appears to be of major clinical interest to decrease lipid levels in diabetic patients with microalbuminuria, not only to retard atherosclerotic manifestations, but also to attenuate possible detrimental effects of high lipid levels on renal function [53].

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