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Frequency Distribution of Apoprotein(a) Isoforms in Patients with Diabetes Mellitus and Healthy Subjects

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Aim. To determine the frequency distribution of apoprotein(a) isoforms in patients with insulin-dependent (IDDM) and non-insulin-dependent (NIDDM) diabetes mellitus and healthy subjects.

Method. We separated and visualized 5 apo(a) isoforms in 40 patients with IDDM (12 men aged 48.00 ± 4.59 and 28 women aged 52.37 ± 8.21), 65 patients with NIDDM (26 men aged 61.88 ± 9.25 and 39 women aged 60.15 ± 7.98), and 182 healthy subjects, using 3-15% gradient sodium dodecyl sulfate polyacrylamide gel electrophoresis, followed by immunoblotting.

Results. The frequency distribution of apo(a) isoforms was very similar in patients with diabetes mellitus and the control group. Atherogenic low molecular weight (LMW) S1 apo(a) isoform was more frequent in patients with IDDM (7.5%) and NIDDM (6.15%) than in the control group (0.78%). LMW S1 apo(a) isoform in patients with IDDM (relative risk [RR], 6.86; 95% confidence interval [CI], 1.19-25.21; $p < 0.001$) and patients with NIDDM (RR, 7.04; 95% CI, 1.40-35.40; $p = 0.0057$) as well as high molecular weight > S4 apo(a) isoform in patients with NIDDM (RR, 2.39; 95% CI, 1.28-5.21; $p = 0.0067$) significantly increased the risk for the development of atherosclerosis. Mean molecular weight of S3, S1, and B apo(a) isoforms was higher in patients with IDDM and NIDDM than in the healthy subjects carriers of the same isoforms, but this difference was not statistically significant. We estimated high inverse statistical correlation between apo(a) size (kDa) and plasma lipoprotein(a) concentration in all study groups, patients with IDDM ($p < 0.001$), patients with NIDDM ($p < 0.001$), and healthy subjects ($p < 0.01$).

Conclusion. Not only the increased plasma Lp(a) levels, but also apoprotein(a) isoforms may play an important role as a risk factor for the development of atherosclerosis in patients with diabetes mellitus.

Kew words: *apoproteins; arteriosclerosis; diabetes mellitus, insulin-dependent; diabetes mellitus, non-insulin- dependent; gene frequency; Macedonia (Republic)*

Apoprotein(a) [apo(a)] is a highly glycosylated, hydrophilic, multikringle protein that has very low affinity for lipids. It circulates as a free particle and as a major component of apoprotein B that contains lipoprotein(a). The primary structure of apo(a) shows a high degree of sequence homology to plasminogen (PLG). McLean et al (1) showed that apo(a) gene is a member of the plasminogen gene superfamily. Both genes, the apo(a) gene and gene that codes plasminogen, are located closely to one another on chromosome 6 (q26-q27). Apo(a) is composed of three distinct structural domains: a single copy of kringle V PLG-like domain; variable number of similar, but not identical kringle IV domains; and inactive serine protease PLG-like domain. Ten units of kringle IV domain are designated as K-IV types 1-10. K-IV type 1 and types 3-10 are present as a single copy in apo(a) particles, whereas type 2 is present in multiple identical 5.6 kb copies, whose number varies between < 3 and > 40 (2). The varying number of K-IV type 2 repeats affects the apo(a) size polymorphism, whose molecular mass varies from ~ 300 to 900 kDa (3). There is an inverse correlation between the apo(a) size (kDa) and plasma lipoprotein(a) concentration (4).

Apo(a) is covalently linked by a disulfide bond to apoprotein B100, a major protein of low density lipoprotein (LDL), and they together form a lipoprotein(a) particle. Lipoprotein(a) is additionally stabilized by extensive hydrogen network (2).

At first, the apo(a) polymorphism was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and later by agarose gel electrophoresis (SDS-AGE) (4,5). SDS-PAGE helped

discern 6 different human apo(a) isoforms based on their relative mobility as compared with apoB100 (4).

It is well known that high plasma lipoprotein(a) levels are an independent risk factor for the development of atherosclerosis in general population (6). Reports on lipoprotein(a) concentration in diabetes are mostly contradictory (6), reporting higher (7), similar (8), or lower (9) lipoprotein(a) levels in patients with diabetes mellitus compared with non-diabetic subjects. Also, a lack of association between high lipoprotein(a) values and coronary hearth disease in diabetic patients, as found by Haffner et al (10), was rebutted by other case control studies (11,12). Many recent studies have paid more attention to that problem, suggesting that apo(a) could play a role independent of lipoprotein(a) levels in the development of coronary heart disease in patients with diabetes mellitus (13-17).

The aim of this study was to determine the frequency distribution of apo(a) isoforms in patients with insulin-dependent and non-insulin-dependent diabetes mellitus (IDDM and NIDDM, respectively) and in healthy subjects in the Republic of Macedonia.

Subjects and Methods

Subjects

We determined apo(a) isoforms in a group of 40 patients with IDDM (12 men aged 48.0±4.6, and 28 women aged 52.4±8.2) and a group of 65 patients with NIDDM (26 men aged 61.9±9.3, and 39 women aged 60.2±8.0), who were diagnosed with the disease at the Diabetes Center of the Clinic of Endocrinology and Metabolism, Medical Faculty, Skopje (Table 1). Diabetes mellitus was diagnosed according to the World Health Organization criteria (fasting glucose ≥7.8 mmol/L and/or glucose level ≥11.1 mmol/L 2 h after glucose load) (18). Diabetic patients who had not been taking insulin were considered to have NIDDM. Forty eight out of 65 patients with NIDDM included in our study took oral hypoglycemic drugs and 17 were on a diabetic diet only. The patients were recruited for the study in the period between September 1999 and April 2001. Individuals with acute inflammatory disease (n=16), neoplastic disease (n=10), and renal insufficiency (serum creatinine levels >90 μmol/L; n=9) were excluded, as well as diabetic patients who had been receiving lipid-lowering therapy (n=23). None of the patients with diabetes mellitus had increased urinary albumin excretion, as determined by Micral® test strips (Roche Diagnostics Ltd., Bell Lane, East Sussex, UK).

Table 1. Characteristics of diabetic patients with insulin-dependent (IDDM) and non-insulin dependent diabetes mellitus (NIDDM), and healthy subjects

	Group		
Variable	IDDM	NIDDM	healthy subjects
No. of subjects	40	65	182
Sex (men/women)	12/28	26/39	89/93
Age (years)	50.2±6.4	60.9±8.3	40.6±8.1
Present smokers (%)	15.6	20.0	16.0
Arterial hypertension (%)	25	23.1	–
Previous AMI* (n)	–	4	–
Duration of diabetes (years)	7.9±4.9	11.0±6.0	–
Fasting glucose (mmol/L)	10.1±3.3†	9.2±2.7†	4.9±0.8
HbA1c (%)	8.5±2.3‡	7.8±1.6‡	5.0±0.5
Lipoprotein(a) (mg/dL)	18.3±14.8§	17.9±17.5§	14.0±10.6
Lipoprotein(a) ≥ 30 mg/dL (%)	20.0	15.5	9.4

*AMI – acute myocardial infarction.
†p≤0.002 vs healthy subjects, Student's t-test.
‡p≤0.05 vs healthy subjects, Student's t-test.
§ p≤0.036 vs healthy subjects, Student's t-test.

The control group comprised 182 healthy volunteer blood donors, (89 men and 93 women aged 40.6±8.1 years), who had donated blood many times (Table 1). The blood donors were recruited from the Republic Institute of Transfusiology, Skopje.

Hypertension was diagnosed if the patient had increased blood pressure level (systolic blood pressure >160 mm Hg, or diastolic blood pressure >90 mm Hg) or if on antihypertensive therapy. Hypertension was found in 10 patients with IDDM and 15 patients with NIDDM. None of the control subjects had hypertension or received hypolipidemic agents. Smoking habit was also recorded: 6 patients with IDDM, 13 patients with NIDDM, and 29 control subjects were present smokers. The rest of the diabetic patients did not declare ex-smoker status.

Information on patient and family history, smoking, dietary supplements, and drug intake was obtained by questionnaires in the presence of a medical doctor. Written informed consent for participation in the study was obtained from all patients with diabetes mellitus. The project was approved by the Ethics Committee at the Doctor's Chamber of the Republic of Macedonia.

Methods

Venous blood was obtained by venipuncture and collected into vacutainers with anticoagulant after a 12-h overnight fast. K3-etylen diamin tetra acetat (EDTA) plasma was separated by low-speed centrifugation (1,800 rpm/min) for 15 min, subdivided into several small samples, and immediately frozen and stored at -80 °C until analysis.

The apo(a) isoforms were determined by 3-15% gradient sodium dodecyl sulfate denaturing polyacrylamide gel electrophoresis (SDS–PAGE) on a vertical Mini-Protean II Bio-Rad System (Bio-Rad Laboratories, Hercules, CA, USA) according to the procedure described elsewhere (19,20). All chemicals and reagents were purchased from Bio-Rad Laboratories and Sigma Chemical Co. (St Luis, MO, USA).

The first step in the analysis was casting 12 mini 3-15% gradient gels with two peristaltic pumps (Masterflex L/S, Cole Parmer Instrument Co., Vernon Hills, IL, US), which were controlled by Cole Parmer software to achieve the gradient characteristics of the gels. In the second step, plasma samples and the standard (Immuno-AG, Vienna, Austria) were treated with SDS-reducing buffer and then subjected to electrophoresis in 3-15% gradient gels. After transfer to nitrocellulose membranes (S&S NC; BA83; 0.2 mm) by Hoefer TE22 Transfer Unit (Amersham-Pharmacia Biotech GmbH, Vienna, Austria), the apo(a) bands were visualized immunochemically with lipoprotein(a) phenotyping kit (Immuno-AG, Technoclone GmbH, Vienna, Austria). According to their relative electrophoretic mobilities on SDS-PAGE as compared with ApoB100, apo(a) isoforms were designated as B, S1, S2, S3, and S4 and divided into respective double-band phenotypes, as previously described by Utermann et al (4). The phenotype was defined as "null" when the blots showed no bands at all (Fig. 1).

The molecular weights of different apo(a) bands were estimated by comparing their mobilities with those of the apo(a) bands in the standard run in an immediately adjacent lane. For this purpose, we used Pharmacia-Biotech Laser scanner with Im-

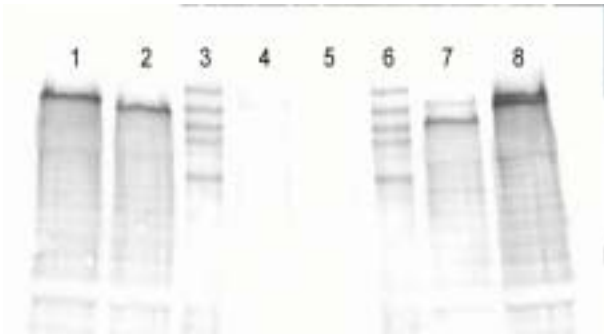

Figure 1. Apoprotein(a) isoforms transferred on a nitrocellulose membrane. Lines 1,2,4,5,7, and 8 refer to plasma samples obtained from patients with diabetes mellitus, and lines 3 and 6 to an apo(a) standard.

age Master Software. In double-banded samples, the molecular weight of the dominant band was estimated for the statistical evaluation of the molecular mass of apo(a) (21).

Lipoprotein(a) concentration was determined at the Department of Immunobiology and Human Genetics by using immunonephelometrical kits (Dade-Behring Marburg GmbH, Marburg, Germany) on a Behring Nephelometer Analyzer. The fasting blood glucose was analyzed by glucose oxidation method on Beckman Glucose Analyzer (Beckman Instruments, Palo Alto, CA, USA) at the Diabetes Center.

Albuminuria was checked in the first morning urine with Micral® test stips (Roche Diagnostics). Each test strip contains monoclonal antibodies against human albumin (IgG) labeled with colloidal gold.

Statistical Analysis

Data were presented as mean±standard deviation (SD). Pearson's correlation coefficient (r) and multiple regression analysis were performed to analyze the association between apo(a) size and lipoprotein(a) concentration. The comparison of molecular weight mean values of different apo(a) isoforms between diabetic patients and healthy subjects was done by Tukey honest significant difference (HSD) test for unequal N (Spjotvoll/Stoline test). As there was no difference in apo(a) isoform frequency distribution between men and women in the three groups of subjects (20), the data for both sexes were presented together.

Statistica® 5.0A for Windows (Stat Soft Inc., Tulsa, OK, USA) was used for all data analyses. The relative risk and confidential intervals were estimated by EpiInfo 6, Version 6,4 b, 97 programs. $P<0.05$ was considered statistically significant.

Results

Frequency Distribution of Apo(a) Isoforms

The distribution of the detected single- and double-banded apo(a) isoforms and the null alleles in both groups of diabetic patients was similar to those in healthy subjects (Fig. 2). The single-banded apo(a) isoforms were frequent in patients with NIDDM (42 of 65) and the control group (105 of 182). The double-banded apo(a) isoforms were present in almost 50% of patients with IDDM (19 of 40). Null phenotype was more frequent in healthy population (11 of 181), as expected (Table 2).

Table 2. Frequency distribution of apoprotein(a) isoforms in patients with insulin-dependent (IDDM) and non-insulin dependent diabetes mellitus (NIDDM) and healthy subjects

Apoprotein(a) isoforms	No. (%) of patients		
	IDDM	NIDDM	healthy subjects
Single	20 (50.0)	42 (64.6)	106 (58.2)
Double	19 (47.5)	20 (30.8)	65 (35.7)
Null	1 (2.5)	3 (4.6)	11 (6.0)
Total	40 (100.0)	65 (100.0)	182 (100.0)

In all subjects, we visualized the following isoforms: >S4, S4, S3, S1, and B. The high molecular weight >S4 apo(a) isoform was more frequent in patients with NIDDM (27.6%), whereas high molecular weight S4 apo(a) isoform was more frequent in patients with IDDM (25.0%) and healthy subjects (32.0%). The low molecular weight S1 apo(a) isoform was more frequent in patients with IDDM (7.5%) and NIDDM (6.2%), compared with only 0.8% in the control group.

Regarding the double-banded apo(a) isoforms in the patients with diabetes mellitus, the following isoforms were detected: >S4S4, >S4S3, S4S4, S4S3, S4S1, S4B, S3S1, and S3B. Among the double-banded

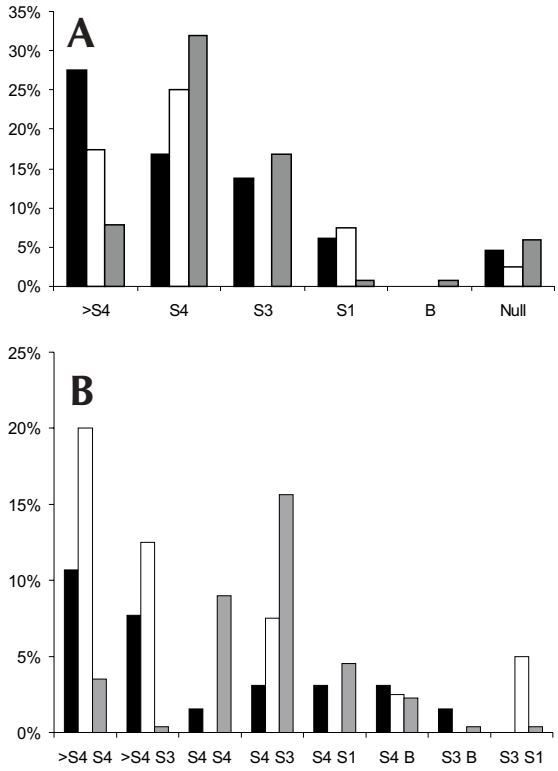

Figure 2. Percentage frequency distribution of (**A**) single-banded and (**B**) double-banded apoprotein(a) isoform in patients with insulin-dependent diabetes mellitus (IDDM, open bars), non-insulin-dependent diabetes mellitus (NID DM, closed bars), and healthy subjects (gray bars).

high molecular weight apo(a) isoforms in both groups of patients, the most frequent was >S4S4 in patients with IDDM (20.0%) and those with NIDDM (10.7%). The double-banded S4S3 apo(a) isoform was most common (15.6%) in healthy population. As far as the double-banded isoforms with at least one low molecular weight apo(a) band are concerned, S4B (2.5%) and S3S1 (5.0%) were more frequent in patients with IDDM and S4B (3.1%) and S3B (1.5%) in patients with NIDDM, as compared with the control group (Fig. 2).

The estimated relative risk of apo(a) isoforms in the development of atherosclerosis in diabetic patients showed that the low molecular weight S1 statistically increased the risk for patients with IDDM (relative risk [RR], 6.86; 95% confidence interval [CI], 1.19-39.74; $p<0.001$) and those with NIDDM (RR, 7.04; 95% CI, 1.40-35.40; $p<0.01$). The statistically significant relative risk of 2.59 (95% CI, 1.28-5.21; $p<0.01$) was also found for high molecular weight >S4 apo(a) isoform, but only in patients with NIDDM.

Molecular Mass of Apo(a) Isoforms

Although larger on average, the molecular mass of all apo(a) isoforms (S4, S3, S1, and B) was not statistically significant in diabetic patients as compared with that of the matched apo(a) isoforms in the control group (Table 3).

Table 3. Molecular mass (kDa) of apoprotein(a) isoforms in patients with insulin-dependent (IDDM) and non-insulin-dependent diabetes mellitus (NIDDM) and healthy subjects

	Molecular mass (mean ± SD)		
Isoform	IDDM	NIDDM	healthy subjects
>S4	792.25 ± 30.07	787.25 ± 31.77	782.50 ± 16.07
S4	721.10 ± 20.15	704.08 ± 35.97	710.48 ± 25.19
S3	607.50 ± 20.27	602.60 ± 29.30	588.05 ± 24.00
S1	527.00 ± 22.89	532.00 ± 15.20	520.00 ± 10.00
B	–	456.00 ± 2.83	429.33 ± 18.00

We found significant inverse correlations of apo(a) size (kDa) and lipoprotein(a) concentrations in both diabetic groups of patients ($r = -0.6503$, $p < 0.001$ for IDDM; and $r = -0.4407$, $p < 0.001$ for NIDDM) and healthy patients ($r = -0.459$, $p < 0.01$) (Fig. 3).

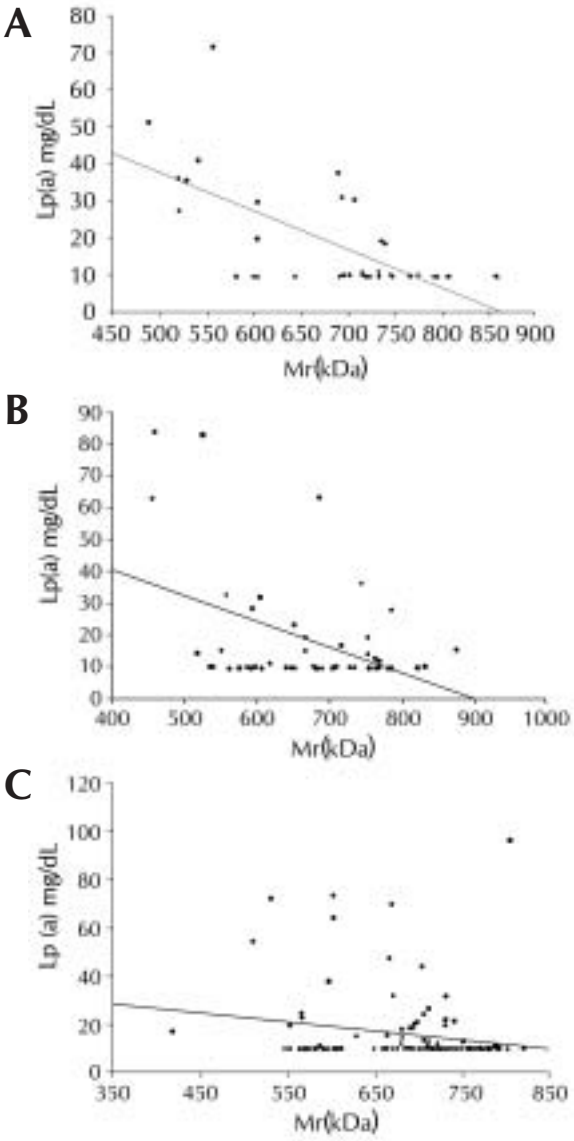

Figure 3. Correlation between apoprotein(a) size (kDa) and lipoprotein(a) concentration (mg/dL) in patients with (**A**) insulin-dependent diabetes mellitus (IDDM), (**B**) non-insulin-dependent diabetes mellitus (NIDDM), and (**C**) healthy subjects.

Discussion

Diabetes mellitus is characterized by an increased risk of atherosclerosis. Apo (a) could play a role in the development of atherosclerosis, regardless of lipoprotein(a) plasma concentration (22-24).

In our previous study, we showed that the carriers of single apo(a) isoform were more frequent among healthy Macedonians (58.2%), especially carriers of high molecular weight S4 and S3 (32.2% and 16.9%, respectively). The low molecular weight B apo(a) isoform was detected in only 2 healthy subjects (20). Concerning the double-banded apo(a) isoforms, the most frequent were high molecular weight S4S3 (15.8%) and S4S4 (9.0%). Double-banded isoforms with at least one low molecular weight band (S4S1, S4B, S3S1, and S3B) were rarely present, as well as low molecular weight single-banded isoforms (S1 and B), as compared with high molecular weight phenotypes. These findings were similar to those reported by other authors (4,5,19,21).

The distribution of apo(a) isoforms found in our diabetic patients was similar to the distribution among healthy Macedonians. This is in accordance with findings by Csaszar et al (8), who found no significant difference in apo(a) isoform distribution in diabetic patients (IDDM or NIDDM) and control subjects from two ethnic groups (Austrians and Hungarians). Gazzaruso et al (25), Ribault et al (26), and Klausen et al (27) found very similar frequency distribution of apo(a) isoforms in patients with IDDM and in healthy individuals. However, Ribault et al (26) reported in the same study a statistically significant difference in the distribution of apo(a) isoform between patients with NIDDM and healthy individuals, as well as between the patients with IDDM and those with NIDDM (26).

Apo(a) phenotype analysis in normoglycemic relatives of patients with IDDM and NIDDM did not point to a difference in the distribution of apo(a) phenotypes between the two groups of patients and their respective healthy relatives (27,28).

Single apo(a) isoforms were more frequent in both groups of patients, but particularly in patients with NIDDM (64.6%). A similar percentage was found by Ruiz et al (10) and Ribault et al (26).

As it could be expected, the null phenotype was more present in the control subjects (6.0%) than in the diabetic patients (4.62% in patients with NIDDM and 2.5% in those with IDDM). Hernandez et al (29) speculated that diabetic patients who carried null phenotype should be considered at low risk for the development of cardiovascular disease.

Our study showed that the low molecular weight single S1 and the double-banded isoform with at least one low molecular weight isoform S4B, S3B, or S3S1 were more frequent in diabetic patients than in healthy individuals. Ribault et al (26) observed a higher prevalence of low molecular weight apo(a) isoforms only in patients with NIDDM.

Another study also showed that the low molecular weight S1 in both groups of diabetic patients, as

well as the high molecular weight >S4 apo(a) isoform in patients with NIDDM, significantly increased the risk in the development of atherosclerosis (30). Gazzaruso et al (17) showed that apo(a) polymorphism in patients with NIDDM, as well as microalbuminuria, homocysteine, and lipoprotein(a) were independent predictors of asymptomatic coronary artery disease in these patients. Large studies showed that the smallest apo(a) isoform was associated with an increased risk for the development of atherosclerosis (23,24,31). We found that high molecular weight >S4 statistically increased the relative risk, but we could not explain the way in which this isoform participated in the development of atherosclerosis in patients with NIDDM. Neither Lundstam et al (32) could explain why high molecular weight S4 apo(a) isoform increased the risk for the total and heart mortality in patients with coronary heart disease.

The molecular masses (kDa) of all apo(a) isoforms were higher in diabetic patients than in the control group. Rainwater et al (9) reported that individuals with NIDDM had significantly larger apo(a) size (kDa) than their non-diabetic relatives carrying genetically identical apo(a) isoforms. This can be explained with the non-enzymatic glycosilation of plasma proteins and therefore of apo(a) glycoprotein in diabetic subjects (33,34). Doucet et al (33) speculated that glycosilated lipoprotein(a) stimulates the formation of cholesterol ester in human macrophages, compared with the native lipoprotein(a), and that the levels of glycosilated lipoprotein(a) in patients with diabetes mellitus correlated with HbA1c.

We found an inverse relationship between apo(a) size and lipoprotein(a) concentration in both groups of patients, NIDDM and IDDM, as well in the controls. This inverse correlation has also been found in many other studies (4,10,26,31).

In conclusion, we have found a similar frequency distribution of apo(a) isoforms in diabetic patients and in healthy subjects. The low molecular weight single-banded apo(a) isoforms and those double-banded with at least one low molecular weight apo(a) band were more frequent in patients with diabetes mellitus than in healthy subjects. The estimated relative risk showed that the apo(a) phenotype might be an independent risk factor in the appearance and development of atherosclerosis in diabetic patients.

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