DETERMINATION OF LIPOPROTEIN(a) 
CONCENTRATIONS AND APOLIPOPROTEIN(a) 
MOLECULAR WEIGHTS IN DIABETIC PATIENTS

A. RIBAULT (1), M.R. DUROU (1), C. LETELLIER (1), F. WOJCIK (1), J.Y. POIRIER (2), A. RUELLAND (1)

SUMMARY
- Lipoprotein(a) (Lp(a)) with atherogenic and thrombotic properties has been frequently studied in diabetes, because a high cardiovascular risk has been reported both in type 1 and type 2 diabetes. Few studies have considered genetic factors, especially the isoforms of apolipoprotein(a). The aim of this work is to determine the distribution of apo(a) phenotypes in the serum of 148 diabetic patients (59 type 1, 89 type 2) with or without vascular complications. Apo(a) phenotypes are determined using 4-15% sodium dodecyl sulfate polyacrylamide gel electrophoresis followed by immunoblotting (PhastSystem – Pharmacia). An inverse relationship is observed between Lp(a) serum concentration and the apparent molecular mass of apo(a) isoforms: type 1 \( r = -0.61, p < 0.01 \); type 2 \( r = -0.55, p < 0.01 \). The frequency of apo(a) isoforms is significantly different between type 1 and type 2 diabetes mellitus. A higher prevalence of isoforms of low molecular weight was observed in the type 2 diabetic population.

Key-words: type 1 diabetes, type 2 diabetes, apo(a) phenotypes.

RÉSUMÉ - Concentrations de lipoprotéine (a) et poids moléculaires d'apoprotéine (a) chez les patients diabétiques.
- La lipoprotéine (a) (Lp(a)) aux propriétés athérogènes et antithrombolytiques a été très étudiée chez des patients diabétiques (type 1 et type 2) car le risque vasculaire au sein de cette population est élevé. Peu d'études ont pris en compte les facteurs génétiques, et notamment l'étude du phénotype de l'apolipoprotéine (a). L'objectif de ce travail est de déterminer la distribution du phénotype de l'apo (a) chez 148 patients diabétiques (59 patients de type 1, 89 patients de type 2) Les isoformes de l'apo (a) sont obtenues après électrophorèse sur gel de gradient de polyacrylamide (4-15 %) en milieu SDS, suivi d'un immunoblotting (système PhastSystem — Pharmacia). Une relation inverse est retrouvée entre la concentration de la Lp(a) et le poids moléculaire de l'apo (a); type 1 \( r = -0.61, p < 0.001 \); type 2 \( r = -0.55, p < 0.01 \). La fréquence des isoformes de l'apo (a) est significativement différente entre la population diabétique de type 1 et celle de type 2. Une plus grande fréquence des isoformes de bas PM est retrouvée chez les patients diabétiques de type 2.

Mots-clés : diabète de type 1, diabète de type 2, phénotypes de l'apo(a).
The lipoprotein(a) (Lp(a)) was first described in human plasma by Berg (1963) as a genetic variant of low-density lipoprotein (LDL). Lp(a) is an LDL-like particle which contains, in addition to apo B100, a disulfide-linked molecule of a protein known as apolipoprotein(a) (apo(a)) which carries the Lp(a) antigen. Apo(a) shares a remarkable structural homology with the fibrinolytic protein zymogen, plasminogen. Apo(a) is formed by three distinct structural domains: an inactive protease domain, a copy of kringle V domain, both of which exhibit 85% homology with the corresponding domains of plasminogen, and multiple copies of the plasminogen-like kringle IV domain. The number of sequence repeats in a given apo(a) molecule is genetically controlled and accounts for the considerable intra and inter-individual size heterogeneity of apo(a). Apo(a) is under the control of a single gene locus localized at the long arm of chromosome 6 (6q2.6-q2.7). The alleles at this locus determine a genetic size polymorphism of apo(a). Some authors have described a possible association between the size heterogeneity of apo(a) and the variability of Lp(a) plasma concentration. These two factors would represent risk factors for development of vascular disease [1].

Atherosclerosis occurs earlier and more frequently in diabetic than in non-diabetic subjects and, in fact, is the major cause of mortality in diabetic patients.

In this report we determine Lp(a) concentrations and investigate the distribution of apo(a) isoforms in diabetic patients and in controls.

SUBJECTS

Subjects in the present study were Caucasian diabetic patients diagnosed according to National data group criteria [2].

Sera of patients with type 1 diabetes were obtained from 59 subjects (32 women, 27 men) aged 18 to 78 years. They displayed ketosis and an absolute requirement for insulin at diagnosis without clinical biological or morphological evidence of secondary diabetes.

Sera of type 2 diabetic patients were obtained from 89 subjects (30 women, 59 men) aged 35 to 87 years. All were on a diet and taking oral hypoglycaemic drugs supplemented with insulin in 58 cases. The main characteristics of the patients are summarized in Table I.

Renal or hepatic insufficiency, thyroid disease, acute and chronic inflammatory disease or infectious state were not observed in any patient. Forty were undergoing lipid-lowering drug treatment. There was no available information regarding oestrogen therapy.

A group of 88 healthy subjects recruited from a health center (43 women and 45 men), mean age 48 +/- 12 years served as the control group.

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Table I. Lipid, lipoprotein and apolipoprotein levels in diabetic patients (type 1, type 2) and controls.

<table>
<thead>
<tr>
<th>Diabetic populations</th>
<th>Type 1</th>
<th>Type 2</th>
<th>Controls</th>
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</thead>
<tbody>
<tr>
<td>N</td>
<td>59 (32 women, 27 men)</td>
<td>89 (30 women, 59 men)</td>
<td>88 (43 women, 45 men)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>45 ± 15 (**)</td>
<td>61 ± 11</td>
<td>48 ± 12</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>24.48 ± 3.87 (**)</td>
<td>29.21 ± 5.62</td>
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<tr>
<td>Diabetes duration (years)</td>
<td>15.4 ± 10.7 (**)</td>
<td>13.20 ± 9.14</td>
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<tr>
<td>Fasting glucose (mM)</td>
<td>11.10 ± 5.15 (**)(*)</td>
<td>9.22 ± 3.30 (*)</td>
<td>8.02 ± 0.34</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>9.58 ± 1.1 (**)</td>
<td>8.05 ± 1.58 (*)</td>
<td></td>
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<tr>
<td>TC (mM)</td>
<td>5.29 ± 1.1 (**)</td>
<td>5.60 ± 1.03</td>
<td>5.38 ± 0.62</td>
</tr>
<tr>
<td>TG (mM)</td>
<td>1.01 ± 0.49 (**)</td>
<td>1.76 ± 0.72 (*)</td>
<td>0.89 ± 0.29</td>
</tr>
<tr>
<td>HDL-C (mM)</td>
<td>1.59 ± 0.43 (**)(*)</td>
<td>1.25 ± 0.46 (*)</td>
<td>1.55 ± 0.27</td>
</tr>
<tr>
<td>LDL-C (mM)</td>
<td>3.33 ± 0.94 (**)</td>
<td>3.63 ± 0.76 (*)</td>
<td>3.25 ± 0.67</td>
</tr>
<tr>
<td>Apo A1 (g/l)</td>
<td>1.64 ± 0.25 (*)</td>
<td>1.59 ± 0.32 (*)</td>
<td>1.87 ± 0.30</td>
</tr>
<tr>
<td>Apo B (g/l)</td>
<td>0.91 ± 0.22 (**)</td>
<td>1.09 ± 0.24</td>
<td>1.03 ± 0.17</td>
</tr>
<tr>
<td>Creatinin (µg/l)</td>
<td>89.70 ± 22.92(**)</td>
<td>98.40 ± 28.42</td>
<td>92.01 ± 20.50</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SD. (*) statistical difference between diabetic patients and controls by Student’s t test (p < 0.001). (**) statistical difference between type 1 and type 2 populations (p < 0.001).
METHODS

Blood samples – Venous blood was collected from the subjects after a 12-hour fast, in vacutainer tubes with or without anticoagulant (EDTA or heparin). After centrifugation (1500 g, 15 min) the sera were analyzed directly. All icteric or hemolytic blood samples were discarded.

Total cholesterol (TC) and triglycerides (TG) were measured in serum with the CHOD-PAP and GPO-PAP kits (Boehringer, Meylan, France) on autoanalyzer Hitachi 717 (Boehringer).

HDL-cholesterol (HDL-C) was measured in serum after precipitation of the lipoproteins that contained apolipoprotein B (LDL, VLDL and Lp(a)) by a phenolphthalein acid-magnesium chloride mixture. LDL-cholesterol (LDL-C) was computed by Friedewald’s formula provided triglycerides were below 4.5 mmol/l [3].

Apolipoproteins A1 and B (apo A1, apo B) were measured in serum by immunonephelometry on autoanalyzer BNIII (Dade Behring, Paris La Défense, France).

Glycated haemoglobin (HbA1c) was determined in whole blood in the presence of EDTA by high performance liquid phase chromatography (HPLC) on Diamat analyzer (Biorad, Ivry sur Seine, France).

Fasting blood glucose was assayed in heparinized plasma using Glucose-PAP kit (Biomerieux).

Lp(a) concentration was determined by an electroimmunodiffusion method according to Laurell [4] in agarose gel with a monoclonal anti apo(a) antibody incorporated in an agarose gel (Hydragel Lp(a) kit, Sebia, Issy-les-Moulineaux, France).

The apolipoprotein (a) phenotype from whole plasma was determined by automated isoelectric focusing using PhastSystem and immunoblotting (Pharmacia France, Saint-Quentin-en-Yvelines, France).

The samples were diluted 1:10 in reducing buffer containing 60 mmol/l Tris, 0.2 mol/l SDS, 0.2% bromophenol blue, 5% β-Mercaptoethanol. 2 μl reduced samples were applied to the gel. Electrophoresis was performed on a 4–5% gradient SDS polyacrylamide gel. Environmental conditions, with a constant gel temperature during electrophoresis, were optimal at 15°C for a total of 63 volthours (Vh) for 30-45 min. The calibration was performed using standard sera. This calculation was made by using the ImageMaster® Software (version 1D) (Pharmacia France).

After extensive washings in Tris buffer (0.1 mol/l, pH 9.5), the apo(a) banding pattern was visualized by chemical staining for alkaline phosphatase.

For the purpose of this study we decided to combine apo(a) phenotypes into two subgroups as proposed by Seed et al. [5]. The first group comprised all subjects displaying a phenotype with at least one small isoform ie below or equal to 650 Kd, size corresponding to the S3 isoform in Utermann’s classification [6, 7]. The second group included all subjects exhibiting one or two apo(a) isoforms above 650 Kd.

In each individual plasma sample, either one or two bands were observed. The molecular weight of each band in the samples was calculated by comparing migration of immediately adjacent bands in the standard sera. This calculation was made by using the ImageMaster® Software (version 1D) (Pharmacia France).

Statistical analysis – Means and standard deviations (means ± SD) were computed for all parameters. The means were compared by Student’s t test, with a p value of 0.05 or less which were considered as significant. The study of correlations between the parameters was performed with a linear regression method. Differences in the frequencies of apo(a) phenotypes between type 1 and type 2 diabetes mellitus were assessed by the chi-square test for contingency tables. The level of significance was set at p < 0.05.

RESULTS

Table I lists the mean values (± SD) for each parameter in the two groups of diabetic patients and in the controls.

Both groups of diabetic patients displayed significantly higher Lp(a) concentrations and lower molecular weight of apo(a) when compared to controls. The means plasma Lp(a) concentration of the patients with type 1 and type 2 diabetes mellitus (respectively 0.34 ± 0.33 g/l and 0.41 ± 0.35 g/l) did not significantly differ. This same result was obtained when we compared the mean values of the molecular weights of apo(a) isoforms (666 ± 88 Kd for type 1 diabetic patients and 657 ± 82 KD for type 2 diabetic patients) (Table II).

No significant correlation was observed between the mean plasma Lp(a) concentration or apo(a) molecular weight and the different lipid and glycaemic parameters.

In both diabetic populations, an inverse relationship between apo(a) size and Lp(a) concentration was found: Type 1 diabetes mellitus r = – 0.61 p < 0.01, Type 2 diabetes mellitus r = – 0.55 p < 0.01.

Each diabetic population was divided into two subgroups in accordance with the apo(a) molecular
weight (below or equal to 650 KD and above 650 Kd). Table III lists the mean values (± SD) for each parameters in the two subgroups. The mean plasma concentrations of the different parameters did not significantly differ between the subgroups except the Lp(a) concentration (type 1: PM ≤ 650Kd Lp(a) = 0.49 ± 0.29g/l, PM > 650Kd Lp(a) = 0.26 ± 0.13 g/l p < 0.0001; type 2: PM ≤ 650 Kd Lp(a) = 0.55 ± 0.32 g/l, PM > 650 Kd Lp(a) = 0.30 ± 0.23 g/l p < 0.0001).

The frequencies of single banded phenotypes as well as those of double-banded phenotypes are shown in figure 1. Among the 148 tested individuals, 99 diabetics (67%) exhibited one isoform (one band) and were classified as homozygous, and 49 patients exhibited two isoforms (two bands) and therefore were classified as heterozygous.

The distribution of the apo(a) phenotypes of diabetic patients and that of control group significantly differed for type 2 diabetes ($\chi^2 = 17.95$, p < 0.01). A significant difference was also observed between type 1 and type 2 diabetes regarding the frequencies of the phenotypes below or equal to 650 KD and above 650 KD ($\chi^2 = 6.93$, p < 0.01).
DISCUSSION

The comparative analysis of the two groups of diabetic patients (type 1 and type 2) does not reveal any significant difference regarding the mean plasma concentration of the Lp(a) and the mean molecular weight of the apo(a), but both groups of diabetics displayed significantly higher Lp(a) concentrations when compared to controls. Forty patients in this study were treated with lipid-lowering drugs, mostly with fibrates. Some reduction of plasma Lp(a) levels might occur in response to therapy with certain lipid modifying agents [8]. In a recent study statins have been reported to raise Lp(a) in some cases [9]. However only a slight modification has been observed and no study demonstrated efficacy in diabetic patients. The general tendency in the literature was to conclude that Lp(a) concentration was elevated in type1 diabetic patients [10, 11]. Recently [12] variations of Lp(a) concentration were studied in a large population of insulin-treated diabetic patients (insulin-dependent diabetic patients and insulin-treated type 2 diabetic patients). The two groups have constantly higher Lp(a) concentrations suggestive of a potential direct or indirect role of exogenous insulin concentration in the modulation of Lp(a) serum concentration. In our population two type 2 diabetic patient out of three were supplemented with insulin.

A significant inverse correlation was found between the Lp(a) plasma concentration and the apo(a) molecular weight in each diabetic group and in the controls. This inverse correlation has also been observed in numerous studies [1, 7, 13, 14]. The variation in the Lp(a) plasma concentration is attributed to the genetically determined isoforms [15, 16]. In a caucasian population, the genetic variations at the apo(a) locus would explain approximately 40% of the variability of the concentration [17, 18].

In our experimental work, 67% of the diabetic patients showed only one type of isoform. This percentage has also been found by Ruiz et al. (63.8%) [13].

Some publications have studied the distribution pattern of apo(a) phenotype in diabetes mellitus in contrast to non-diabetic control subjects. As in our study, Gazzaruso et al. [19] have found, no significant difference in apo(a) isoforms frequencies between type 1 diabetes mellitus and control population. As opposed to our results, Császár et al. [20] have shown that the distribution of the apo(a) phenotypes of type 2 diabetes mellitus and that of control group are not different, suggesting that the phenotype of the apo(a) is independent of diabetes, but the two ethnically different populations studied by these authors were well-controlled type 2 diabetic patients with no insulin treatment. No publication has studied the comparison of the apo(a) phenotypes distribution between type 1 and type 2 diabetes mellitus. Our present results suggest that apo(a) isoforms frequencies are significantly different between type 1 and type 2 diabetic patients. A higher prevalence of isoforms of low molecular weight is observed in type 2 diabetic population. Linden et al. [21] have reported that the low relative molecular mass isoforms (B, S1, S3) are significantly overrepresented in the coronary disease patients (p = 0.0003) in contrast with a control population. Ruiz et al. [13] have demonstrated a positive association between elevated Lp(a) levels and coronary heart disease in type 2 diabetic patients, an association which is partially accounted for by the higher frequency of apo(a) isoforms of small size. High Lp(a) levels and apo (a) isoforms of low relative molecular weight seem to be associated with the presence of proliferative retinopathy [22]. The results of these studies suggest that the apo(a) phenotypes of low molecular weight seem to influence the risk of cardiovascular complications, but large, long-term, prospective studies are required to confirm a possible relation between the apo(a) phenotype and the incidence of vascular complications in diabetes mellitus.

CONCLUSION

Our study demonstrates that the distribution of apo(a) phenotypes significantly differs between type 1 and type 2 diabetes mellitus. Type 2 diabetic patients...
show a higher frequency of low molecular weight isoforms of apo(a). However other studies would be needed to confirm the relationship between the apo(a) phenotypes distribution and the presence of vascular complications in diabetes mellitus.

REFERENCES


