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Is apolipoprotein(a) a susceptibility gene for Type I diabetes mellitus and related to long-term survival?

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Abstract

Aims/hypothesis. High lipoprotein(a) [Lp(a)] plasma concentrations are a genetically determined risk factor for atherosclerotic complications. In healthy subjects Lp(a) concentrations are mostly controlled by the apolipoprotein(a) [*apo(a)*] gene locus which determines a size polymorphism with more than 30 alleles. Subjects with low molecular weight *apo(a)* phenotypes on average have higher Lp(a) concentrations than those with high molecular weight *apo(a)* phenotypes. There are many opinions about whether and why Lp(a) is raised in patients with Type I diabetes (insulin-dependent) mellitus.
Methods. We investigated Lp(a) plasma concentrations and *apo(a)* phenotypes in 327 patients with Type I diabetes mellitus (disease duration 1–61 years) and in 200 control subjects matched for age and sex.
Results. Patients with a disease duration of up to 15 years had significantly higher Lp(a) concentrations (24.3 ± 34.0 mg/dl vs 16.7 ± 22.6 mg/dl, $p = 0.014$) compared with control subjects. This increase can be explained by a considerably higher frequency of low molecular weight *apo(a)* phenotypes (38.9 % vs 23.5 %, $p < 0.005$). The frequency of low molecular weight *apo(a)* phenotypes decreased continuously with disease duration from 41.7 % in those with disease duration of up to 5 years to 18.2 % in those with the disease lasting more than 35 years.
Conclusion/interpretation. Our data show that an increase of Lp(a) in Type I diabetic patients can only be observed in groups with short diabetes duration and that this elevation is genetically determined. Therefore, the *apo*(*a*) gene, located at 6q26–27, might be a susceptibility gene for Type I diabetes mellitus which is supported by recently published studies reporting evidence for linkage of this region (6q27) with Type I diabetes mellitus. Furthermore, the decreasing frequency of low molecular weight *apo(a)* phenotypes with disease duration suggests a survivor effect. [Diabetologia (1999) 42: 1021–1027]

Keywords Type I diabetes mellitus, *apo*(*a*) gene, *apo(a)* polymorphism, Lp(a), susceptibility gene, atherosclerosis, risk factor.

High lipoprotein(a) [Lp(a)] plasma concentrations have been reported to be a risk factor for coronary artery disease in most prospective studies [1]. Concentrations of Lp(a) are determined strongly by the apolipoprotein(a) [*apo*(*a*)] gene locus and correlate with a size polymorphism of *apo(a)*. The molecular basis for this size polymorphism is a varying number of kringle-IV (K-IV) copies which are highly homologous to the K-IV of plasminogen. There exists an inverse correlation between the number of K-IV repeats of *apo(a)* and the Lp(a) plasma concentrations. Subjects with a low number of K-IV repeats on average have high concentrations of Lp(a) and those with a high number of K–IV repeats show low concentrations. Investigations in the general population

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Abbreviations: *apo(a)*, apolipoprotein(a); Lp(a), lipoprotein(a); K-IV, kringle-IV; HMW, high molecular weight; LMW, low molecular weight.

found that the *apo(a)* gene locus at 6q26–27 co-determines the risk for coronary heart disease through its allelic control of Lp(a) plasma concentration [2–8].

There is extensive discussion whether Lp(a) is raised in patients with Type I diabetes mellitus [1, 9]. Raised [10–12] and similar [12–17] concentrations compared with control subjects were reported but study groups were heterogeneous in terms of study subjects' age and duration of diabetes. Also the presence of micro- or macroalbuminuria might have influenced the results. Only few studies were originally designed to investigate whether a possible increase of Lp(a) in these patients is primarily associated with diabetes mellitus or whether this increase is secondary to the disease. These studies included *apo(a)* phenotype analysis and came to the conclusion that *apo(a)* is not associated with Type I diabetes mellitus [14, 17]. Recent studies, however, reported evidence for linkage of Type I diabetes mellitus and region 6q27 [18–22] which is within the region were the *apo(a)* gene is located (6q26–27).

Since diabetic patients have a tremendous risk for coronary artery disease [23–25], the group of patients under investigation has to be homogeneous in terms of age and diabetes duration so as not to diminish a possible association by a survivor effect. We therefore evaluated Lp(a) plasma concentrations and *apo(a)* phenotypes in 200 control subjects and 327 patients with Type I diabetes mellitus stratified by diabetes duration to address two questions. Firstly, is the *apo(a)* size polymorphism associated with Type I diabetes mellitus and secondly, is the *apo(a)* size polymorphism associated with long-term survival in these patients.

Subjects and methods

Patients and control subjects. We investigated 327 consecutive and unrelated Type I diabetic patients who were under observation of the outpatients department at the 3rd Medical Department of the Hospital Lainz, Vienna. The study group was recruited from the outpatient clinic during the yearly follow-up programme after informed consent was obtained. The study was approved by the local ethics review board. Inclusion criteria were Type I diabetes mellitus, regular attendance at the outpatient clinic in the last year and stable metabolic control with no episode of ketoacidosis in the last 3 months. Type I diabetes mellitus was defined as diabetes manifestation before the age of 30 years and insulin treatment within 1 year from diagnosis.

The 133 women and 194 men were on average 40.1 ± 12.7 years old and had diabetes for 22.2 ± 12.8 years at the time of inclusion into the study. To compare Lp(a) plasma concentrations and *apo(a)* phenotype frequency, we recruited from the same geographical region a group of 200 healthy voluntary blood donors frequency matched for age and sex to the group of patients with disease duration up to 15 years.

Laboratory measurements. Patients' blood was drawn after an overnight fast. Creatinine and HbA_{1c} (normal value < 6%) were analysed immediately. For other biochemical variables EDTA plasma was frozen and kept at –80 °C before analysis [26].

Lp(a) quantification was done as described in detail [26] with a double-antibody ELISA using an affinity-purified polyclonal *apo(a)* antibody for coating and the horseradish peroxidase-conjugated monoclonal 1A2 for detection. This anti-*apo(a)* antibody recognises the epitope motif YYPN in kringle IV (K–IV) type 2 [27]. An Lp(a) positive serum from Immuno (Vienna, Austria) with the same *apo(a)* isoforms served as standard throughout the whole study. Each sample was analysed in duplicate, and intra- and interassay coefficients of variation were 2.7% and 6%, respectively.

Apo(a) phenotyping was done by sodium dodecyl sulphate-agarose gel electrophoresis (SDS agarose) [28] under reducing conditions, as outlined previously [29], with some minor modifications [30]. With this procedure it was possible to detect two *apo(a)* isoforms in 60% of the subjects under investigation. In only five subjects (0.9%) we were not able to detect any *apo(a)* isoform. *Apo(a)* phenotyping in patients and control subjects was done at the same time and the investigators were kept unaware of the duration of diabetes mellitus.

Urinary albumin excretion was measured from an overnight sample using the turbidometric method in patients with known albustix negative urine or microalbuminuria. Patients were classified into three categories: normal albuminuria (below 20 μg/min), microalbuminuria (20–200 μg/min) and macroalbuminuria (> 200 μg/min). In patients with macroalbuminuria or raised creatinine a 24-h urine sample was collected to determine 24-h protein excretion. To exclude ongoing urinary infections, sediment examination was done routinely in a spontaneously voided urine sample in all patients.

Statistical analysis. Since we hypothesised an influence of diabetes duration with survival, we grouped patients in three groups according to the tertiles of diabetes duration. Lp(a) plasma concentration of patients were adjusted for HbA_{1c} using multivariate regression analysis. Comparisons of Lp(a) concentrations between patients (whole group and group with short diabetes duration) and control subjects were done by the nonparametric Wilcoxon rank sum test; Lp(a) concentrations between patients of different diabetes duration were compared by the Kruskal-Wallis test. We used the Mantel-Haenszel test for linear association to compare the frequencies of *apo(a)* phenotypes between diabetic patients in the tertile with long and short duration of diabetes mellitus. The frequency of LMW *apo(a)* phenotypes between the different groups was compared using the Pearson's χ^2 test. We did not correct for multiple comparisons, since we had decided a priori to compare only the patient group with short diabetes duration with the control group and not the patient group with middle or long disease duration. The comparison of the whole patient group with control subjects was done additionally to show the effect of heterogeneity in terms of diabetes duration on the results.

Because of the high number of detectable *apo(a)* isoforms (> 30), many phenotypes were only represented in low numbers (e. g. once). To account for this problem and to get sufficient sample sizes in each category, we decided a priori to combine *apo(a)* isoforms in steps of three K–IV repeats and to categorize phenotypes according to the molecular weight of the smaller *apo(a)* isoform [31]. Since phenotypes with 11–16 or more than 34 K–IV repeats in the smaller *apo(a)* isoform were underrepresented, we formed one group by combining 11–19 and one by combining more than 31 K–IV repeats. In a further step, we divided *apo(a)* phenotypes in two subgroups according to the molecular weight of the smaller *apo(a)* iso-

Table 1. Lp(a) plasma concentrations and *apo(a)* size polymorphism in control subjects and patients with Type I diabetes mellitus. Results in patients are given separately by stratification in tertiles of diabetes duration

Characteristics	Control subjects *(n = 200)*	All patients *(n = 327)*	Duration of Type I diabetes mellitus (years)		
			1–15 *(n = 108)*	16–27 *(n = 112)*	28–61 *(n = 107)*
Age, years; mean ± SD (median)	29 ± 12 (28)	40 ± 13 (38)	31 ± 9 (29)	39 ± 9 (38)	51 ± 10 (52)
Mean diabetes duration, years; mean ± SD (median)		22.2 ± 12.8 (22.0)	8.2 ± 4.4 (8.5)	21.8 ± 3.6 (23)	36.6 ± 7.8 (34.0)
Lp(a), mg/dl; mean ± SD (median)[a]	16.7 ± 22.6 (6.8)	19.1 ± 26.1 (8.6)[b]	24.3 ± 34.0 (9.9)[c,d]	15.4 ± 18.2 (8.6)[d]	17.6 ± 22.8 (6.9)[d]
Apo(a) phenotypes defined by the smaller *apo(a)* allele, *n,* (%)[e,f,g]					
11–19 K-IV repeats	14 (7.0)	24 (7.3)	12 (11.1)	6 (5.4)	6 (5.6)
20–22 K-IV repeats	33 (16.5)	66 (20.2)	30 (27.8)	18 (16.1)	18 (16.8)
23–25 K-IV repeats	30 (15.0)	54 (16.5)	13 (12.0)	24 (21.4)	17 (15.9)
26–28 K-IV repeats	44 (22.0)	73 (22.3)	21 (19.4)	27 (24.1)	25 (23.4)
29–31 K-IV repeats	36 (18.0)	57 (17.4)	14 (13.0)	21 (18.8)	22 (20.6)
> 31 K-IV repeats	43 (21.5)	53 (16.2)	18 (16.7)	16 (14.3)	19 (17.8)
Apo(a) phenotype groups					
LMW[k] *apo(a)* phenotypes, *n,* (%)	47 (23.5)	90 (27.5)[h]	42 (38.9)[i]	24 (21.4)	24 (22.4)[j]
HMW[l] *apo(a)* phenotypes, *n,* (%)	153 (76.5)	237 (72.5)	66 (61.1)	88 (78.6)	83 (77.6)
HbA_{1c}, %; mean ± SD (median)	–	8.7 ±1.4 (8.5)	8.8 ± 1.6 (8.5)	8.6 ± 1.3 (8.6)	8.6 ± 1.4 (8.4)
Microalbuminuria, *n,* (%)	–	23.5	11.1	22.3	37.4
Proteinuria, *n,* (%)	–	12.5	1.9	9.8	26.2

[a] Lp(a) plasma concentrations in patients are adjusted for HbA_{1c}.
[b] $p = 0.22$ by Wilcoxon rank sum test for comparison of Lp(a) plasma concentrations between control subjects and all patients.
[c] $p = 0.014$ by Wilcoxon rank sum test for comparison of Lp(a) plasma concentrations between patients with short disease duration (1–15 years) and control subjects.
[d] $p = 0.17$ by Kruskal-Wallis-test for comparison of Lp(a) plasma concentrations of the three patient groups with different diabetes duration.
[e] Pearson's χ^2 test comparing the frequencies of *apo(a)* phenotypes of all Type I diabetic patients with control subjects: $\chi^2 = 3.03$, $df = 5$, $p = 0.70$.
[f] Mantel-Haenszel test for linear association comparing the frequencies of *apo(a)* phenotypes between Type I diabetic patients with short disease duration (1–15 years) and control subjects: $\chi^2 = 5.8$, $df = 1$, $p = 0.016$.
[g] Mantel-Haenszel test for linear association comparing the frequencies of *apo(a)* phenotypes between Type I diabetic patients with long (28–61 years) and short (1–15 years) disease duration: $\chi^2 = 4.1$, $df = 1$, $p = 0.042$.
[h] Pearson's χ^2 test comparing the frequencies of LMW *apo(a)* phenotypes of Type I diabetic patients with control subjects: $\chi^2 = 1.04$, $df = 1$, $p = 0.31$.
[i] Pearson's χ^2 test comparing the frequencies of LMW *apo(a)* phenotypes of Type I diabetic patients with short disease duration (1–15 years) with control subjects: $\chi^2 = 8.08$, $df = 1$, $p = 0.004$.
[j] Pearson's χ^2 test comparing the frequencies of LMW *apo(a)* phenotypes between patients with long (28–61 years) and short (1–15 years) disease duration: $\chi^2 = 6.84$, $df = 1$, $p = 0.009$.
[k] including all subjects with at least one *apo(a)* isoform with 11 to 22 K-IV repeats.
[l] all subjects who had only isoforms with more than 22 K-IV repeats.

forms, as done in all of our and some of others previous works [3, 4, 8, 30, 32, 33]. The low molecular weight group (LMW) included all subjects with at least one *apo(a)* isoform with 11 to 22 K–IV repeats [29]; the high molecular weight (HMW) group comprised all subjects who had only isoforms with more than 22 K–IV repeats. In case two *apo(a)* isoforms were detectable, we used only the smaller *apo(a)* isoform for categorization [3, 4, 8, 30, 32, 33].

Statistical analysis was done with Statistical Package for the Social Sciences (SPSS) for Windows 7.5.2.

Results

Comparison of Type I diabetic patients and control subjects. First, we compared Lp(a) plasma concentrations and the *apo(a)* phenotype distribution between the whole group of diabetic patients and the control subjects. Lp(a) was slightly raised in patients when compared with control subjects without reaching significance (19.1 ± 26.1 mg/dl vs 16.7 ± 22.6 mg/dl, $p = 0.22$). The *apo(a)* phenotype frequency was also not significantly different in terms of number of K-IV repeats or in terms of LMW *apo(a)* phenotypes (Table 1).

Since the patient group showed a wide range in the duration of diabetes mellitus from 1 to 61 years, we stratified the group in tertiles according to disease duration (1–15 years, 16–27 years and > 27 years). We compared the frequency of the *apo*(*a*) alleles of the group with a short duration of diabetes (1–15 years) with the control group to see whether Type I diabetes mellitus is associated with the *apo*(*a*) size polymorphism. Both groups were comparable for sex (58.2 % vs 56.0 % men) and age (30.7 ± 9.2 years vs 29.1 ± 12.0). Lp(a) was higher in patients with short diabetes duration compared with control subjects (24.3 ± 34.0 mg/dl vs 16.7 ± 22.6 mg/dl, $p = 0.014$). We also observed a pronounced difference in the

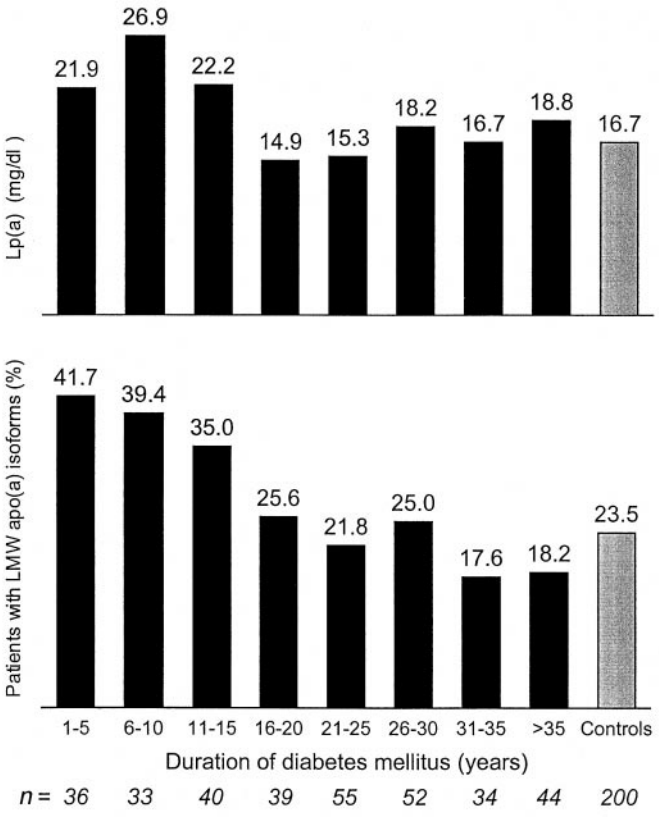

Fig. 1. Lp(a) plasma concentrations and frequency of LMW *apo(a)* isoforms in Type I diabetic patients in relation to disease duration. The frequency of LMW *apo(a)* isoforms clearly decreased with duration of diabetes mellitus ($p = 0.001$, Mantel-Haenszel test for linear association). *n* indicates the number of subjects in each group. Results are also given for control subjects (grey bars)

apo(*a*) allele frequency which was caused by a preponderance of LMW *apo(a)* isoforms in patients with the short diabetes duration (38.9 % vs 23.5 % in control subjects, $p = 0.004$) (Table 1). Statistical calculations showed that we had 77 % power at a 95 % confidence level to detect this difference which is only 3 % below the conventional level.

Relation to diabetes duration. We then investigated a possible survivor effect in relation to the Lp(a) plasma concentrations and the *apo*(*a*) size polymorphism. The Lp(a) concentrations tended to be lower in groups with longer duration of diabetes mellitus (Fig. 1). This trend, however, did not reach significance probably due to a distinctively higher frequency of microalbuminuria and proteinuria in patients with long diabetes duration (Table 1). The *apo*(*a*) size polymorphism in terms of LMW versus HMW *apo(a)* phenotypes showed a clearly decreasing frequency of LMW *apo(a)* phenotypes with the duration of diabetes mellitus ($p = 0.001$, Mantel-Haenszel test for linear association). This frequency decreased from 41.7 % in those with 1–5 years disease duration to 18.2 % in those with more than 35 years of disease duration (Fig. 1). The disease duration was significantly shorter in patients with LMW *apo(a)* phenotypes compared with those with HMW phenotypes (18 ± 12 years vs 24 ± 12 years, $p < 0.001$).

To test this relationship in more detail, we compared the *apo(a)* phenotype frequencies between patients in the tertiles with short (1–15 years) and long duration (> 27 years) of diabetes. The frequency of LMW *apo(a)* phenotypes was considerably lower in patients with long compared with those with short diabetes duration (22.4 % vs 38.9 %, $p = 0.009$) which indicates a disadvantage in long-term survival for patients with LMW *apo(a)* isoforms (Table 1).

To exclude that the decreasing frequency of LMW *apo(a)* isoforms with diabetes duration is simply an effect of age, we calculated the frequency of LMW *apo(a)* isoforms in the different age groups of our and of a previous control group [33]. In both control groups the frequency of LMW *apo(a)* isoforms did not change with age.

Discussion

Siblings of subjects with Type I diabetes mellitus have an about 15 times higher risk for diabetes mellitus than random subjects. Although the major histocompatibility complex (MHC) was found to be the major susceptibility locus for Type I diabetes mellitus, explaining 34 % of the familial clustering of the disease [34], several other susceptibility loci scattered throughout the genome were described [35]. One of these loci called IDDM 8 is assigned to the chromosomal region 6q27 [18–22] which is within the region where the *apo*(*a*) gene is located (6q26–27) [36–38]. Therefore, *apo*(*a*) represents a very likely candidate gene for Type I diabetes mellitus.

We report for the first time that the *apo*(*a*) size polymorphism might be directly associated with Type I diabetes mellitus. We observed a noticeably higher frequency of patients with short duration of diabetes and LMW *apo(a)* isoforms when compared with control subjects frequency matched for sex and age. This preferential association of LMW *apo(a)* isoforms with diabetes indicates that the *apo*(*a*) gene could actually be a susceptibility gene for Type I diabetes mellitus. The alternative explanation is a linkage disequilibrium with a susceptibility locus for Type I diabetes mellitus near the *apo*(*a*) gene locus on chromosome 6q26–27 [36].

We were able to find this difference in *apo(a)* allele frequency only when we investigated patients stratified in groups according to diabetes duration. This might be of considerable importance since Lp(a) plasma concentrations and the *apo*(*a*) size polymorphism are possibly related to survival due to the association with atherosclerotic complications.

We observed a pronounced and nearly linear decrease of the frequency of LMW *apo(a)* phenotypes with diabetes duration (Fig. 1). Patients with a disease duration up to 5 years showed an LMW *apo(a)* phenotype frequency of 41.2% in comparison with only 18.2% in patients with more than 35 years of disease. It is well documented by several studies of the general population that the *apo(a)* gene locus determines the risk for coronary heart disease through its control of Lp(a) plasma concentration [2–8]. A recent study described a pronounced association between LMW *apo(a)* phenotypes and coronary heart disease. This association was strongest for patients with coronary heart disease with younger age at onset [39]. We showed in a prevalence study including 607 haemodialysis patients [30] and in a prospective study in 440 haemodialysis patients [40] that especially *apo(a)* isoforms of low molecular weight and the presence of diabetes mellitus are associated with an increased risk of major cardiovascular events. There is the possibility of a negative impact of LMW *apo(a)* phenotypes on the survival of patients with Type I diabetes mellitus, aged about 35 to 40 years and older. It is in accordance with the decreased frequency of LMW *apo(a)* phenotypes we observed after a disease duration of about 20 years and more and the observation that the mortality rate due to coronary heart disease rapidly increases after age 30 years [23–25]. Lp(a) plasma concentrations did not show such a pronounced decrease with disease duration as one would expect from the decreasing frequency of LMW *apo(a)* phenotypes. This might be explained by the increasing microalbuminuria and proteinuria with disease duration which were shown to increase Lp(a) concentrations [13, 41–44].

Two large studies that investigated Lp(a) in diabetic children found Lp(a) concentrations to be significantly raised in patients compared with control children [10, 11] both of similar age or puberty stage. Studies in adult Type I diabetic patients described mostly unchanged Lp(a) concentrations [13–17]. The young age and short duration of diabetes in children might be the reason that a clear increase of Lp(a) was still detectable, because a cardiovascular survival effect at this age is negligible. Adult patients in most of the above studies were on average diabetic for a longer time than our group with the short diabetes duration of 1 to 15 years (average 8.2 ± 4.4 years). These patients would not be expected to differ much from control subjects if a survivor effect is present which decreases the prevalence of LMW *apo(a)* isoforms with time. Therefore, studies including older patients with longer duration of diabetes might have missed an association of Lp(a) plasma concentrations or *apo(a)* isoforms with diabetes mellitus or both. A further argument against an increase of Lp(a) concentrations in Type I diabetic patients was a study of identical twins which found very similar Lp(a) concentrations in diabetic patients and their healthy co-twins [45]. This observation, however, rules out diabetes as a secondary but not primary cause of high Lp(a) in Type I diabetic patients. Since identical twins share *apo(a)* alleles identical by descent, a genetic contribution to the increase of Lp(a) in Type I diabetic patients cannot be detected by such a study design.

A recent in vitro investigation observed that insulin suppresses the *apo(a)* synthesis in primary cultures of cynomolgus monkey hepatocytes [46]. This is in line with some studies describing a relation between poor glycaemic control in Type I diabetic patients and high Lp(a) plasma concentrations [12, 47, 48]. We can rule out, however, that the increase of Lp(a) is secondarily caused by the changed hormonal status in our patients, since *apo(a)* phenotyping clearly showed a higher frequency of LMW *apo(a)* phenotypes in patients with short disease duration. We furthermore adjusted Lp(a) for HbA_{1c} values and could therefore exclude that, besides this primary effect, a secondary effect due to glycaemic control has influenced our results.

This study is a cross-sectional study with the possibility of bias. To avoid a selection bias we included consecutive and unrelated patients. Nevertheless, it is conceivable that more patients with atherosclerotic complications were attending the routine check-ups at the outpatient department. If that were the case it would increase the frequency of patients with LMW *apo(a)* phenotypes especially in the group with higher age and longer diabetes duration. Therefore, a dilution of the observed survivor effect for patients with HMW *apo(a)* isoforms would be the consequence. We would not expect that atherosclerotic complications are a major reason for younger patients with a short disease duration to visit the outpatient department.

Our analysis is based on *apo(a)* phenotypes derived from plasma proteins and not from genomic analysis. We do not think, however, that this has influenced our findings since *apo(a)* phenotyping derived from plasma and from genomic DNA show a high correspondence [29]. This is especially true for LMW *apo(a)* isoforms which are usually accompanied with high Lp(a) concentrations and a high detection probability in SDS agarose gel electrophoresis.

In summary, these results suggest that LMW *apo(a)* phenotypes are associated with Type I diabetes mellitus and further that these isoforms are associated with a decreased probability of survival in patients with a long duration of diabetes mellitus.

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