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Relationship among Urinary Albumin Excretion Rate, Lipoprotein Lipase *PvuII* Polymorphism and Plasma Fibrinogen in Type 2 Diabetic Patients

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Summary

Plasma fibrinogen level represents a strong cardiovascular risk factor and is regulated by an interplay of genetic and environmental factors. Hyperfibrinogenemia frequently occurs in cluster with dyslipidemia within the frame of insulin resistance syndrome (IRS) and type 2 diabetes mellitus. Genetic variants with a pleiotropic effect have been proposed to cause IRS features including hyperfibrinogenemia. We studied the influence of polymorphisms in lipoprotein lipase (*LPL*) gene, β-fibrinogen gene (*FIBB*) and environmental factors on plasma fibrinogen levels in type 2 diabetes patients. 131 type 2 diabetes patients (mean age 62 ± 10 years, 33 % male) were genotyped for polymorphisms in *LPL* gene (intron 6 *PvuII*, intron 8 *HindIII*) and *FIBB* gene (*-148C/T*, *-455G/A*) by PCR-RFLP method. Fibrinogen was measured by thrombin coagulation method, albuminuria by immunoturbidimetric assay. Polymorphism *LPL PvuII* showed a gene-dose effect on fibrinogen levels, with the highest fibrinogen in *P-P-* homozygotes (p = 0.05, analysis of variance). *P*-carriers (*P-P-* and *P+P-* combined) had significantly higher fibrinogen levels compared with *P+P+* homozygotes (3.74±1.40 g/l vs 3.06±1.20 g/l, p=0.03). Other studied polymorphisms were not significantly related to fibrinogen levels. Age- and sex-adjusted fibrinogenemia correlated significantly with albuminuria (r = 0.48, p=0.001), serum uric acid (r = 0.42, p=0.006) and serum creatinine (r = 0.32, p=0.04). Multiple stepwise linear regression identified interaction term of *LPL PvuII* and albuminuria as an independent predictor of fibrinogen level, explaining 18 % of fibrinogen variance. Albuminuria thus appears to be the best predictor of fibrinogen plasma levels in type 2 diabetic patients. Relationship between albuminuria and fibrinogenemia may be modified by the genotype *LPL PvuII,* which also shows a weak association with plasma fibrinogen level in type 2 diabetes patients.

Key words

Type 2 diabetes mellitus • Fibrinogen • Albuminuria • Lipoprotein lipase gene polymorphisms • Gene-environment interaction

Introduction

Elevated plasma fibrinogen level is a strong and widely acknowledged cardiovascular risk factor in both diabetic and non-diabetic populations (Maresca *et al.* 1999, Howard *et al.* 2000). Playing a crucial role in many

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atherothrombosis-associated processes (hemostasis-fibrinolysis, inflammation, platelet aggregation, blood viscosity, smooth muscle proliferation and migration), it has also been proposed to account for a part of the excessive cardiovascular risk in type 2 diabetic patients that remains unexplained after considering the traditional risk factors (Maresca *et al.* 1999). Notwithstanding its recognized value as a marker for the presence of vascular disease, it remains controversial whether fibrinogen is in a causal relationship or merely in association with the atherosclerotic process (van der Bom *et al.* 1998).

Fibrinogen production in the liver is regulated by cytokines, mainly by interleukin-6 (IL-6), and is greatly enhanced by the acute phase response to inflammatory processes (Vasse *et al.* 1996). Hence, fibrinogen elevation might simply reflect the low-grade inflammation associated with vascular disease. On the other hand, increased fibrinogen levels (due to inflammation or other mechanisms) may still participate in the pathogenesis of vascular lesions, i.e. be a true modifier of the atherosclerotic disease and contribute to its progression. Moreover, fibrinogen and fibrin degradation products might in turn enhance the inflammatory aspect of vascular lesions by regulating cytokine production and leukocyte-endothelial interactions (Flick *et al.* 2004).

The clinically most important and prevalent conditions associated with both elevated fibrinogen level and cardiovascular disease are type 2 diabetes mellitus and the closely related insulin resistance syndrome (IRS) which affect 10-25 % of the general population in developed countries (Imperatore *et al.* 1998, Boulogne and Vantyghem 2004). However, the mechanisms leading to hyperfibrinogenemia in insulin-resistant and type 2 diabetic subjects have not been elucidated so far, even though a potential role of low-grade inflammation, hyperinsulinemia and albuminuria has been discussed (Zanetti *et al.* 2001, Barazzoni *et al.* 2003, Yudkin *et al.* 2004). Furthermore, the phenomenon of clustering of features within IRS and the high heritability of its components, have led some researchers to propose the existence of "insulin resistance genes" with a pleiotropic effect on metabolism. These genes are supposed to induce changes in multiple metabolic traits with the subsequent development of IRS features including hyperfibrinogenemia (de Lange *et al.* 2003).

Lipoprotein lipase has emerged as a physiological candidate for the putative insulin resistance gene. Indeed, a considerable body of evidence has accumulated from epidemiological, experimental and transgenic animal studies supporting the role of lipoprotein lipase and its gene in the pathogenesis of insulin resistance. This evidence is primarily derived from the central physiological role of LPL in the lipoprotein and fatty acid metabolism. Furthermore, a transgenic animal model showed, for instance, that overexpression of LPL improves insulin resistance in rabbits on a high-fat diet (Kitajima *et al.* 2004). A genome-wide linkage analysis and several genetic association studies found a relationship between the region harboring LPL gene on chromosome 8 (8p22) or its polymorphisms, respectively, and insulin resistance syndrome components (dyslipidemia, obesity, hypertension, microalbuminuria) or surrogate indices of insulin sensitivity/resistance (Cai *et al.* 2004, Goodarzi *et al.* 2004). Recently, a compelling evidence of both linkage and association of haplotypes of LPL gene with a direct index of insulin sensitivity has been provided (Goodarzi *et al.* 2004).

Importantly, despite the high heritability of fibrinogen plasma levels (30-50 %) estimated in twin and family studies, common polymorphisms of its gene explain only a negligible part of the fibrinogen level variance (Freeman *et al.* 2002). This discordance implies existence of additional regulatory genes as well as a potential role for interaction among genetic and environmental factors in determining fibrinogen level.

Hence, the aim of this study was to evaluate the influence of selected genetic polymorphisms of β-fibrinogen gene and lipoprotein lipase gene (putative insulin resistance gene), as well as environmental factors on the plasma level of fibrinogen in type 2 diabetic patients. Furthermore, we aimed to investigate the presence of potential interactions between genetic factors and diabetic environment such as glycemic control and albuminuria in determining fibrinogen levels.

Methods

Present study included 131 consecutive type 2 diabetes mellitus patients hospitalized at the Department of Medicine IV of University Hospital in Košice, Slovakia between 2000-2002. Patients with a history of type 2 diabetes mellitus, as defined by the American Diabetes Association, and the absence of any disease known to affect fibrinogen levels such as acute or chronic inflammatory disease, malignancy, hepatic disease, end-stage renal disease, acute myocardial infarction or stroke

within three months before the inclusion into the study, were eligible for this study. The studied patients were not on any lipid lowering treatment affecting fibrinogen levels such as fibrates. The study protocol was approved by the Hospital Ethics Committee. Mean age of the study participants was 62±10 years, 33 % were males.

Biochemical measurements

Blood was withdrawn after an overnight fast. Fibrinogen was determined using thrombin coagulation method (Pacific Hemostasis, USA). Blood glucose, serum creatinine, uric acid, lipids and albumin were measured using routine biochemical assays and glycated hemoglobin was measured using immunoturbidimetric assay (Roche Diagnostica, France). Urinary albumin excretion (UAE) from 24-h urine collection was determined using radial immunodiffusion assay. The categories of normo-, micro- and macroalbuminuria refer to UAE less than 20 mg/l, 20-199 mg/l and UAE above 200 mg/l, respectively.

Genotype determination

DNA was isolated from leukocytes of frozen EDTA-treated whole blood by a high salting-out method and amplified by polymerase chain reaction (PCR) in a Perkins Elmer/Cetus Thermal Cycler. To amplify a 1.3-kb fragment containing the *HindIII* restriction site in intron 8 and a 858-bp fragment containing the *PvuII* restriction site in intron 6 of the *LPL* gene, we used a modification of the method described by Ahn *et al.* (1993). The modifications for the *HindIII* site were 0.9 μmol/l each primer and 35 cycles. The modifications for the *PvuII* site were 0.5 μmol/l each primer, annealing at 67 °C and 33 cycles. For determination of β-fibrinogen gene (*FIBB*) polymorphisms *–148C/T* and *–455G/A* (restriction sites of HaeIII and HindIII, respectively) we used a modification of method described by Rupert *et al.* (1999) with forward primer 5´ AAG AAT TTG GGA ATG CAA TCT CTG CTA CCT 3´ and reverse primer 5´ CTC CTC ATT GTC GTT GAC ACC TTG GGA 3´. Blind samples (reagents minus DNA) were included in each PCR reaction to exclude contamination. Amplified products were digested overnight at 37 °C with appropriate restriction enzyme according to the manufacturer's recommendations (Boehringer Mannheim). After separation on 2 % agarose gels, the resulting fragments were stained with ethidium bromide and visualized with an UV transilluminator. The alleles with the restriction site and the non-cleavable alleles were designated *P+* and *P-* for the *LPL PvuII, H+* and *H-* for *LPL HindIII*, *G* and *A* for the *FIBB -455G/A* (*HaeIII)* and *C* and *T* for the *FIBB –148C/T (HindIII)* polymorphism, respectively. From the whole sample 120, 120, 124, 117 participants have been successfully genotyped for *FIBB –148C/T* and *–455G/A*, *LPL HindIII* and *PvuII* polymorphisms, respectively.

Statistical analysis

Continuous variables are presented as means ± SD, except for urinary albumin excretion (UAE) where median, 25th and 75th percentile are shown. Natural logarithmic transformation of variables with skewed distribution was used in the analysis (UAE, triglycerides, fibrinogen). Means or medians of untransformed data are presented in tables for better comprehension. Analysis of variance (ANOVA) with Bonferroni´s correction for multiple testing was used for comparison of the means across the genotypes. Student´s t-test was applied to compare means between two groups. Hardy-Weinberg equilibrium as well as distribution of the categorical variables across genotypes was examined by a χ^2-test. Pearson correlation coefficients adjusted for sex and age were calculated to assess relationships between continuous variables. Multiple stepwise linear regression models were used in the multivariate analysis to reveal the independent predictors of fibrinogen level. Potential interactions between independent variables were examined by introducing interaction terms into the multivariate models. The values of $p<0.05$ were considered statistically significant. Statistical programs Sigmastat v. 2.0 and SPSS v. 10 were used for the analyses.

Results

The study group consisted of 131 patients with type 2 diabetes mellitus (age 62±10 years, BMI 30±5 kg/m^2) with predominance of women (67 %), mostly postmenopausal (85 % of women). The clinical and biochemical characteristics of patients according to *LPL PvuII* genotype are presented in Table 1.

The distributions of the studied genotypes were the following: ***FIBB –455G/A***: *GG* 58.3 %, *GA* 36.7, % *AA* 5.0 %; ***FIBB –148C/T***: *CC* 56.7 %, *CT* 38.3 %, *TT* 5.0 %; ***LPL PvuII*****:** *P+P+* 20.6 %, *P+P-* 53.8 %, *P-P-* 25.6 %; ***LPL HindIII*****:** *H+H+* 35.5 %, *H+H-* 56.5 %, *H-H-* 8.0 %. Distribution of genotypes did not deviate significantly from the Hardy-Weinberg equilibrium

(HWE) in any of the polymorphisms.

Across the genotype groups of *LPL PvuII* polymorphism, the mean fibrinogen plasma level did rise concomitantly with the number of *P*- alleles in the genotype, indicating thus the presence of a gene-dose effect. As a result, *LPL PvuII P-P-* homozygotes had the highest mean fibrinogen level, with borderline statistical significance in the analysis of variance (Table 1). Further, carriers of *P*- allele of *LPL PvuII* polymorphism (*P+P-* and *P-P-*) were combined and their mean fibrinogen level was compared with the homozygotes *P+P+* in the analysis of pooled genotypes. Plasma fibrinogen was significantly higher in the pooled group of *P*- carriers in comparison with the *P+P+* group (3.74±1.40 g/l vs 3.06± 1.20 g/l, p = 0.03; Student´s t-test). The mean fibrinogen level did not differ significantly across genotype groups in none of the other studied polymorphisms, neither in the ANOVA test nor in the analysis of pooled genotypes (data not shown). As for the *LPL PvuII* polymorphism, there was no significant difference in age, sex, diabetes duration, glycated hemoglobin (HbA1c), smoking habits, body mass index, serum creatinine or albuminuria among the genotype groups (Table 1).

Table 1. Clinical and biochemical characteristics of Type 2 diabetes patients according to *LPL PvuII* genotype

	LPL *PvuII* genotype			
	P+P+	**P+P–**	**P–P–**	**p**
Number of patients	24	63	30	
Age (years)	61 ± 10	61 ± 10	62 ± 9	ns
BMI (kg/m^2)	30.7 ± 5.2	29.8 ± 5.4	30.4 ± 4.3	ns
Sex (% males)	46	25	33	ns
Current smokers (%)	17	22	28	ns
Menopause (% women)	92	80	90	ns
Hypertension (%)	92	84	93	ns
HbA1c (%)	7.2 ± 2.0	7.7 ± 2.3	8.1 ± 2.2	ns
DM duration (years)	8.0 ± 7.8	8.0 ± 6.1	10.5 ± 7.5	ns
Insulin treatment (%)	27	41	50	ns
Fibrinogen (g/l)	3.06 ± 1.20	3.64 ± 1.44	3.93 ± 1.32 *	0.05
Creatinine (μmol/l)	108 ± 55	94 ± 29	100 ± 39	ns
UAE (mg/l)	10; 10; 10	56; 36; 91	365; 265; 810	ns
Uric acid (μmol/l)	333 ± 114	351 ± 117	331 ± 102	ns
Total cholesterol	5.01 ± 1.41	5.50 ± 1.23	5.46 ± 1.18	ns
Triglycerides	2.65 ± 1.43	2.70 ± 1.52	2.69 ± 1.45	ns
LDL-C	2.88 ± 1.08	3.39 ± 1.05	3.51 ± 1.18	ns
HDL-C	1.00 ± 0.34	0.99 ± 0.31	0.82 ± 0.25	ns
Apolipoprotein B	1.02 ± 0.29	1.11 ± 0.26	1.09 ± 0.27	ns

* *P-P- vs P+P+*, p < 0.05, Data shown as mean ± SD, except for UAE – median; 25 %; 75 %, *BMI* – body mass index, *DM* – diabetes mellitus, *HbA1c* – glycated hemoglobin, *HDL-C* – high density lipoprotein cholesterol, *LDL-C* – low density lipoprotein cholesterol, S – serum, *UAE* – urinary albumin excretion, *WHR* – waist-to-hip ratio, *ns* – not significant

Linear correlation coefficients between fibrinogen and other variables are shown in Table 2. After an adjustment for sex and age, fibrinogen showed a significant positive correlation with urinary excretion rate, uric acid, and serum creatinine level. Across the categories of UAE (normo-, micro-, macroalbuminuria) there was a significant increase of plasma fibrinogen (g/l; 3.09±0.97, 3.47±1.18, 4.37±1.86, ANOVA p = 0.008) and decrease of albuminemia (g/l; 43.1±6.5, 40.7±5.3, 36.8±3.6, ANOVA p = 0.001), respectively.

Table 2. Age and sex adjusted partial correlation coefficients between fibrinogen and other variables (n = 120)

	r	p
UAE	0.48	0.001
Serum uric acid	0.42	0.006
Serum creatinine	0.32	0.04
BMI	0.09	ns
HbA1c	0.05	ns
Serum albumin	-0.17	ns

BMI – body mass index, *HbA1c* – glycated hemoglobin, *UAE* – urinary albumin excretion rate, *ns* – not significant

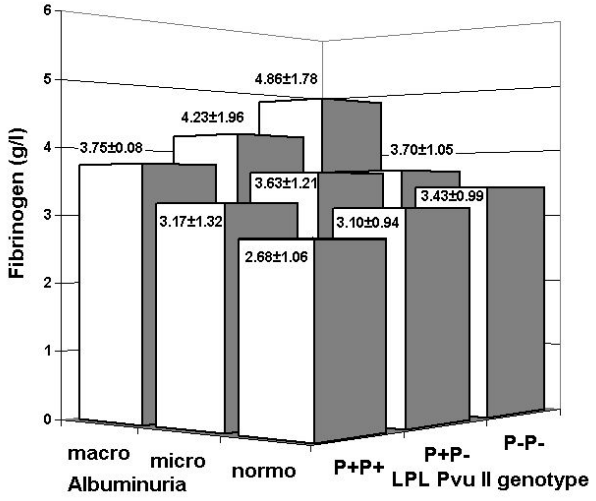

Fig. 1. Relationship among *LPL PvuII* genotype, category of albuminuria (UAE) and plasma fibrinogen level. Multiple linear regression selected interaction term *LPL PvuII* * UAE as the best predictor of fibrinogen level (p<0.001), explaining 18 % of its variance.

Multiple linear regression analysis was carried out to determine independent predictors of fibrinogen level. With fibrinogen as a dependent variable, included in the multivariate model were all variables that showed association with fibrinogen level in the univariate analysis (UAE, *LPL PvuII* genotype, creatinine, uric acid) and those that could potentially affect the fibrinogen level according to the physiological knowledge from literature (age, sex, HbA1c, BMI, smoking status).

Urinary albumin excretion (UAE), entered as a categorical variable with values for normo-, micro- and macroalbuminuria, was the only independent predictor of plasma fibrinogen selected in the forward stepwise linear regression model (P<0.001, n = 84). UAE accounted for 12 % of fibrinogen variance. When interaction term of *LPL PvuII* * UAE was added into the model, it replaced the UAE as the best independent predictor of fibrinogen level (P < 0.001), augmenting the proportion of explained fibrinogen variance to 18 %. Figure 1 depicts the relationship among UAE category, *LPL PvuII* genotype and plasma fibrinogen.

Other studied polymorphisms showed no significant interaction with albuminuria. Similarly, there was no significant interaction between any of the studied polymorphisms and glycated hemoglobin as a measure of long-term glycemic control (data not shown).

Discussion

Our findings in the present study show that in patients with type 2 diabetes, plasma fibrinogen level may be best predicted by urinary albumin excretion rate, and that this relationship may be modified by the *PvuII* polymorphism of the lipoprotein lipase gene, a putative gene for insulin resistance.

Hence, our results suggest a presence of gene-environment interaction between *LPL PvuII* genotype and albuminuria in determining fibrinogen levels in diabetic patients. Moreover, our data indicate a possible association between the *LPL PvuII* genotype and fibrinogen level, given the presence of a gene-dose effect and a significant elevation of fibrinogen in *P*- carriers compared with *P+P+* homozygotes.

Association between fibrinogen and albuminuria in type 2 diabetes patients has been previously reported by several authors (Festa *et al.* 2000, Tkáč *et al.* 2003). Two recent studies also found a significant association between LPL polymorphisms *PvuII* or *HindIII* and microalbuminuria in type 2 diabetic patients (Mattu *et al.* 2002, Solini *et al.* 2004). Albuminuria could thus act as a physiological mediator or a statistical confounder of LPL polymorphism-fibrinogenemia relationship. However, in our study we found no significant difference in the magnitude of albuminuria among the *LPL PvuII* genotype groups.

Although the correlation between fibrinogenemia and albuminuria could be attributed to their common association with the inflammation underlying the insulin resistance syndrome, diabetes, vascular and renal disease, there might also exist another pathophysiological link between these two variables. Notably, in patients with nephrotic range or near-nephrotic range of proteinuria urinary albumin loss with

subsequent hypoalbuminemia has been shown to stimulate hepatic proteosynthetic rate of plasma proteins including fibrinogen (Zanetti *et al.* 2001). This pathway has been postulated to act through decreased plasma oncotic pressure as a primary stimulus for the induction of proteosynthesis. On the other hand, it is not clear whether the outlined mechanism also applies to patients with microalbuminuria and non-nephrotic low-grade proteinuria.

Interestingly, our cross-sectional data were consistent with the proposed concept of albuminuria-hypoalbuminemia-hyperfibrinogenemia relationship and we could observe a significant decrease of serum albumin levels with a concomitant increase of fibrinogen levels across the categories of normo-, micro- and macroalbuminuria, respectively.

Given the evidence from studies of liver-specific gene expression, it is a conceivable and biologically plausible notion that the regulation of fibrinogen hepatic synthesis in response to environmental stimuli such as hypoalbuminemia and decreased plasma oncotic pressure due to albuminuria is governed by a large network of genes and transcription factors (Schrem *et al.* 2002). Hence, our observation of genotype-environment interaction between *LPL PvuII* polymorphism and albuminuria may imply that intronic *LPL PvuII* polymorphism might be involved in regulation of fibrinogen plasma levels.

In view of the fact that LPL gene, a putative insulin resistance gene, is not expressed in the liver its direct influence on fibrinogen genetic transcription is improbable. Still, its potential association with fibrinogen transcription and plasma level might be mediated either through linkage disequilibrium with other causal gene e.g. with the transcription factor or indirectly, on the biochemical level, through some of the pleiotropic pathophysiologic actions of its product – lipoprotein lipase protein in extrahepatic tissues (Merkel *et al.* 2002).

There are several limitations of our study that are common to most genetic association studies concerning in particular relatively small sample size, potential confounding variables and population stratification bias (Colhoun *et al.* 2003). Multiple testing as a source of random associations has been postulated to affect genetic association studies and may also apply to our study. To minimize the impact of multiple comparisons, we used a Bonferroni *post hoc* test, one of the accepted methods of adjustment in the analysis of variance. To eliminate the effect of potential confounding variables we performed multivariate analysis. Furthermore, small sample size with subsequently small power to detect an effect of minor magnitude may have contributed to our failure to replicate the previously described association of fibrinogen level with β-fibrinogen gene polymorphisms (Colhoun *et al.* 2003).

In this study, we have demonstrated that in type 2 diabetic patients, the presence of a gene-environment interaction between albuminuria and *LPL PvuII* genotype in predicting plasma fibrinogen levels, while this prediction is predominantly mediated by albuminuria. Furthermore, we found a weak association between *LPL PvuII* polymorphism and plasma fibrinogen. These findings, however, require confirmation and further testing in a more stringent physiological model. A recently proposed novel study approach offered by the systems biology integrating data from multiple fields of biological research (genomics, proteomis, metabolomics, transgenic animals etc.) may yield new original insight into the regulation of fibrinogen (Ghazalpour *et al.* 2004).

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