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Elevated plasma phospholipid transfer protein activity is a determinant of carotid intima-media thickness in type 2 diabetes mellitus

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Abstract *Aim/hypothesis:* The plasma activity of phospholipid transfer protein (PLTP), which has putative pro- and anti-atherogenic roles in lipoprotein metabolism, is increased in type 2 diabetes mellitus. We analysed the relationship between carotid artery intima–media thickness (IMT), an established marker of atherosclerosis, and PLTP activity in diabetic patients and control subjects. *Methods:* The IMT (mean of three segments in both carotid arteries by ultrasonography), clinical variables, plasma PLTP activity (phospholipid vesicle–HDL system), lipoproteins, C-reactive protein and insulin were measured in 87 non-smoking men and women, who had type 2 diabetes mellitus, no cardiovascular disease, and were not on insulin or lipid-lowering medication, and in 83 age-matched control subjects. *Results:* In diabetic patients, carotid IMT (p=0.02), pulse pressure (p=0.003), plasma PLTP activity (p<0.001), triglycerides (p=0.01), C-reactive protein (p<0.01) and insulin (p<0.001) were higher, whereas HDL cholesterol was lower (p<0.001) than in control subjects. Multiple stepwise linear regression analysis demonstrated that in type 2 diabetic patients IMT was independently associated with age (p<0.001), sex (p=0.001), pulse pressure (p=0.003), plasma PLTP activity (p=0.03) and HDL cholesterol (p=0.03), but not with very low density lipoprotein+LDL cholesterol, triglycerides, C-reactive protein and insulin (all p>0.20). The relationship between plasma PLTP activity and IMT was not significant in control subjects. *Conclusions/interpretation:* Plasma PLTP activity is a positive determinant of IMT in type 2 diabetes mellitus, suggesting that high PLTP activity is involved in accelerated atherosclerosis in this disease.

Keywords C-reactive protein · High-density lipoprotein · Intima–media thickness · Phospholipid transfer protein · Type 2 diabetes mellitus

Abbreviations Apo: apolipoprotein · CRP: C-reactive protein · IMT: intima–media thickness · PLTP: phospholipid transfer protein

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Introduction

The multifunctional role of phospholipid transfer protein (PLTP) in lipoprotein metabolism is increasingly recognised. PLTP facilitates the transfer of phospholipids and free cholesterol from the surface of triglyceride-rich lipoproteins towards HDL particles during intravascular lipolysis [1, 2]. Furthermore, PLTP is able to convert HDL_3 into larger and smaller particles [3], generating small lipid-poor pre-β-HDL particles [4, 5], which may act as initial acceptors of cell-derived cholesterol. Overexpression of PLTP enhances pre-β-HDL formation [6], thereby stimulating cholesterol removal and preventing cholesterol accumulation in macrophages [7]. Besides these potentially anti-atherogenic actions, PLTP also has pro-atherogenic potential, as evidenced by stimulation of hepatic apolipoprotein (apo) B secretion and by effects on HDL levels in mouse models [8–10]. Moreover, PLTP has the

ability to transfer the antioxidant α-tocopherol between lipoproteins, and it has been demonstrated that experimental PLTP deficiency results in an increased α-tocopherol content in LDL, thereby protecting these lipoproteins against oxidation [11].

Experimental findings suggest that the overall effect of PLTP is likely to be pro-atherogenic. The development of atherosclerosis is accelerated in mice overexpressing human PLTP [9, 12], but is attenuated in PLTP knockout models [10]. Little is known about the relationship between plasma PLTP activity and cardiovascular disease in humans. One cross-sectional study demonstrated that the plasma PLTP activity level was higher in patients with coronary artery disease than in subjects recruited from the general population [13].

We and others have previously found that plasma PLTP activity is increased in obese subjects [14–16], and is metabolically linked to triglyceride and NEFA metabolism [14, 15, 17]. Interestingly, several reports have shown that plasma PLTP activity is elevated in type 2 diabetes mellitus [15, 17–19], although most of these studies were performed in small numbers of subjects. Taken together, these findings suggest that high plasma PLTP activity could contribute to increased atherosclerosis in type 2 diabetic patients.

The present study was initiated to test the hypothesis that in patients with type 2 diabetes mellitus the level of plasma PLTP activity is a determinant of carotid intima–media thickness (IMT), an accepted marker of subclinical atherosclerosis [20].

Subjects, materials and methods

Subjects and procedure

The study protocol was approved by the local medical ethics committee of the University Medical Center Groningen and written informed consent was obtained from each participant. Type 2 diabetic patients and age-matched non-diabetic control subjects were recruited by advertisement in local newspapers. Type 2 diabetes mellitus was previously diagnosed using blood glucose thresholds as defined by the WHO. Only non-smoking subjects aged over 18 years were included. Subjects currently or previously taking lipid-lowering drugs were excluded from participation in order to prevent bias due to the effects of such medication on lipoprotein metabolism as well as on IMT progression. Insulin treatment was also an exclusion criterion. None of the participants had clinically manifest cardiovascular disease, and the urinary albumin concentration was below 20 mg/l in all of them. In diabetic patients, treatment for hypertension was allowed. Maximal alcohol intake was three drinks per day, a drink being defined as 250 ml beer, 100 ml wine or 35 ml spirits.

All participants were evaluated after an overnight fast at the Laboratory of Vascular Medicine. BMI was calculated as weight (kg) divided by height (m) squared. Waist circumference was measured as the smallest circumference between rib cage and iliac crest. Systolic and diastolic blood pressures were measured after at least 15 min of rest at the left arm in sitting position using a sphygmomanometer. Pulse pressure was calculated as the difference between systolic and diastolic blood pressures.

Carotid IMT measurement

The IMT of the carotid arteries was measured by ultrasonography in the supine position. Well-trained sonographers, who were not informed about the subject's disease state, scanned high-resolution B-mode ultrasound images (128 XP; Acuson, Mountain View, CA, USA) with a 7.5 MHz linear array transducer. Three arterial wall segments in each carotid artery were imaged from a fixed lateral transducer angle at the far wall. The segments scanned were the segment 1 cm proximal to the carotid dilatation (common carotid artery), the segment between the carotid dilatation and carotid flow divider (carotid bulb) and a 1 cm segment distal to the flow divider (internal carotid artery). The scans were recorded on S-VHS tape and analysed off-line by an independent image analyst, who was unaware of the subjects' characteristics. B-mode image analyses were digitised with a frame grabber (DT286 l; Data Translation, Marlboro, MA, USA). The image analysis software was developed using an algorithm as developed by Selzer et al. [21]. The mean IMT over the six segments of both carotid arteries was calculated and was designated mean IMT. At a mean IMT of 0.80 mm, intersonographer variability amounted to 0.05 mm, with variability among image analysts less than 0.03 mm, corresponding to a total variation coefficient of between 6.3 and 7.3%.

Laboratory measurements

Venous blood samples for measurement of apo and PLTP activity were collected into EDTA-containing tubes (1.5 mg/ml) and placed on ice immediately. Plasma was obtained within 30 min by centrifugation at 1,400 *g* for 15 min at 4°C. Samples were kept frozen at −80°C until analysis.

Plasma total cholesterol and triglycerides were assayed by routine enzymatic methods (Roche/Hitachi, catalogue numbers 11876023 and 11875540, respectively; Roche Diagnostics, Mannheim, Germany). HDL cholesterol was determined in the supernatant fraction after precipitation of VLDL+LDL lipids with polyethylene glycol-6000. VLDL+LDL cholesterol was calculated by subtracting plasma HDL cholesterol from plasma total cholesterol. Apolipoprotein A-I and apo B were measured by immunoturbidimetry (Roche/Cobas Integra Tina-quant, catalogue numbers 03032566 and 03032574, respectively; Roche Diagnostics). Plasma PLTP activity was assayed using a phospholipid vesicle–HDL system, as previously described [14, 22], using [^{14}C]-labelled dipalmitoyl phosphatidylcholine. In short, small plasma samples (1 μl) were incubated with [^{14}C]-phosphatidylcholine-labelled phosphatidylcholine vesicles and excess pooled normal

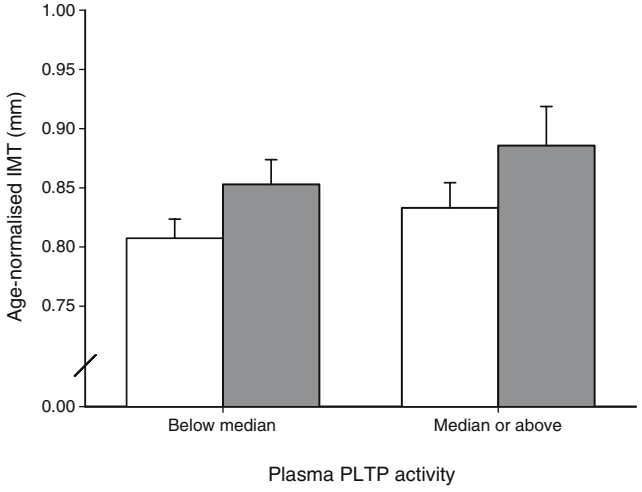

Fig. 1 Age-normalised mean intima–media thickness (IMT) according to plasma phospholipid transfer protein (PLTP) activity in type 2 diabetic patients (*shaded bars*) and control subjects (*open bars*). *Bars* indicate mean±SEM

HDL for 45 min at 37°C. The vesicles were then precipitated using a mixture of NaCl, $MgCl_2$ and heparin (final concentrations: 230 mmol/l, 92 mmol/l and 200 IU/ml respectively). The PLTP activity levels vary linearly with the amount of plasma added to the incubation system. This method is specific for PLTP activity and the phospholipid transfer-promoting property of cholesteryl ester transfer protein (CETP) does not interfere with the assay [22]. Plasma PLTP activity is related to the activity in human reference pool plasma and is expressed in arbitrary units (AU; 100 AU corresponds to 13.6 μmol phosphatidylcholine transferred per litre per hour).

Glucose was analysed with an APEC glucose analyser (APEC, Danvers, MA, USA). Glycated haemoglobin was measured by HPLC (Bio-Rad, Veenendaal, the Netherlands; normal range 4.6–6.1%).

Plasma C-reactive protein (CRP) was determined by nephelometry with a threshold of 0.175 mg/l (BNII N; Dade Behring, Marburg, Germany).

Plasma insulin levels were assessed using a microparticle enzyme immunoassay (AxSYM Insulin assay; Abbott Laboratories, Abbott Park, IL, USA).

Statistical analysis

Parameters with a normal distribution are given as mean± SD. Parameters with a skewed distribution are given as median (interquartile range). Data were compared using unpaired *t*-tests or Mann–Whitney *U*-tests where appropriate. To evaluate differences in proportions of parameters, we used χ^2 analysis. Multiple (stepwise) linear regression analysis was used to disclose independent relationships between variables. When a variable was not normally distributed, logarithmically transformed values of this variable were used in the models. The relative contributions of variables in the regression models were expressed as the IMT effect, which was estimated by multiplying regression coefficient *B* with the SD of the independent variable of interest included in the model, as well as the partial correlation coefficients. The IMT dimension, as shown in Fig. 1, is normalised to the mean age in each group, applying regression coefficients as determined by univariate linear regression analysis. Two-sided *p* values less than 0.05 were considered to be significant.

Results

The study included 87 type 2 diabetic patients and 83 control subjects. There was no difference in sex distribution between the type 2 diabetic and control groups (p=0.47). Among control and diabetic women, 65 and 79%, respectively, were postmenopausal (p=0.18). Median diabetes duration in type 2 diabetic patients was 5.4 years. Patients were treated with diet alone (26%) or in combination with oral glucose-lowering agents (74%). Patients using oral glucose-lowering drugs were treated with sulphonylurea (33%), biguanides (26%) or the combination of

Table 1 Clinical characteristics and mean carotid artery intima–media thickness (IMT) in type 2 diabetic patients and control subjects

	Type 2 diabetic patients n=87	Control subjects n=83	p
Age (years)	57.9±9.0	55.8±9.4	0.15
Sex (M/F)	53/34	46/37	0.47
Diabetes duration (years)	5.4 (4.0–6.4)	NA	–
Mean carotid IMT (mm)	0.876±0.203	0.814±0.144	0.02
Body mass index (kg/m^2)	29.0±5.1	25.7±3.6	<0.001
Waist circumference (cm)	101.1±13.4	88.7±12.9	<0.001
Systolic BP (mmHg)	143.3±19.0	131.8±18.8	<0.001
Diastolic BP (mmHg)	86.8±8.7	82.5±10.7	<0.001
Pulse pressure (mmHg)	56.5±16.5	49.3±13.9	0.003
Fasting glucose (mmol/l)	8.8±2.2	5.6±0.7	<0.001
Plasma insulin (μU/ml)	9.6 (6.7–15.2)	6.0 (4.5–8.4)	<0.001
HbA_1c (%)	6.8±1.0	5.3±0.4	<0.001
CRP (mg/l)	1.74 (1.01–4.23)	1.24 (0.50–2.44)	<0.001

Except for sex, data are mean±SD or median (interquartile range).
NA Not applicable

Table 2 Plasma lipid parameters and phospholipid transfer protein (PLTP) activity in type 2 diabetic patients and control subjects

	Type 2 diabetic patients n=87	Control subjects n=83	p
Plasma total cholesterol (mmol/l)	5.34±0.89	5.74±0.98	0.005
VLDL+LDL cholesterol (mmol/l)	4.09±0.97	4.24±1.05	0.35
HDL cholesterol (mmol/l)	1.35±0.36	1.64±0.42	<0.001
Plasma triglycerides (mmol/l)	1.71 (1.18–2.34)	1.30 (0.88–1.93)	0.01
Plasma apo A-I (g/l)	1.32±0.23	1.43±0.23	0.002
Plasma apo B (g/l)	0.92±0.21	0.96±0.24	0.34
Plasma PLTP activity (AU)	103.4±11.6	94.7±10.0	<0.001

Data are mean±SD or median (interquartile range)

these two (41%). In addition to these drugs, eight patients were also taking a thiazolidinedione and two patients were using acarbose, an α-glucosidase inhibitor. Forty-four per cent of diabetic patients but none of the control subjects used one or more antihypertensive drugs. Twenty-one patients used an ACE inhibitor, either alone (n=9) or in combination with a β-blocker, a calcium-antagonist and/or a diuretic (n=12). Seven patients used an angiotensin II antagonist in combination with other antihypertensive drugs. Ten patients used a β-blocker alone (n=7), a diuretic alone (n=2) or a β-blocker in combination with a calcium antagonist (n=1). As shown in Table 1, age was similar in diabetic patients and control subjects. Body mass index, waist circumference, systolic and diastolic blood pressures, pulse pressure, fasting glucose, HbA_1c, plasma CRP and insulin were all higher in type 2 diabetic patients. The carotid mean IMT was higher in type 2 diabetic patients than in control subjects.

Plasma total cholesterol was slightly lower in type 2 diabetic patients compared with control subjects (Table 2), but VLDL+LDL cholesterol and plasma apo B levels did not differ between the two groups. In type 2 diabetic patients, plasma triglycerides were higher, whereas HDL cholesterol and plasma apo A-I were lower than in control subjects. Plasma PLTP activity was higher in type 2 diabetic patients compared with control subjects (Table 2).

Multiple stepwise linear regression analysis was performed in the combined groups to assess whether plasma PLTP activity was still higher in the diabetic state after controlling for diabetes-related differences in obesity and plasma lipid levels. In this analysis plasma PLTP activity was independently and positively associated with the presence of type 2 diabetes mellitus (p<0.001), waist circumference (p<0.001), HDL cholesterol (p=0.001) and plasma triglycerides (p=0.008, multiple r = 0.56). Plasma PLTP activity was not independently related to plasma insulin (p=0.22), CRP (p=0.72) and VLDL + LDL cholesterol (p=0.99). When diabetic patients were evaluated separately, plasma PLTP activity was positively related to waist circumference (p<0.001), plasma insulin (p=0.003) and HDL cholesterol (p=0.003, multiple r = 0.56), but not with fasting glucose (p=0.19), plasma triglycerides (p=0.42), the use of antihypertensive medication (p=0.52) and VLDL + LDL cholesterol (p=0.55). The positive relationship with HbA_1c was nearly significant (p=0.07).

In each group, multiple stepwise linear regression analysis was also used to evaluate the independent contribution to mean IMT of age, sex, haemodynamic parameters, lipid variables including HDL cholesterol, VLDL+LDL cholesterol (or plasma apo A-I and apo B) and plasma triglycerides, as well as PLTP activity, CRP and insulin. Age and sex are known to be important determinants of IMT [23]. Besides age and sex, systemic haemodynamic factors positively affect IMT [24]. Of the various parameters reflecting blood pressure, pulse pressure was the best-fitting variable in the present study. We therefore included age, sex and pulse pressure in the model when evaluating the relationships of plasma PLTP activity and other laboratory parameters with IMT. The independent determinants of IMT in each group are shown in Tables 3 and 4. In type 2 diabetic patients, plasma PLTP activity was a positive determinant of IMT independent of age, sex, pulse pressure and HDL cholesterol (multiple r=0.71), whereas there were no independent relationships of IMT with plasma CRP (p=0.18), insulin (p=0.24), VLDL+LDL cholesterol (p=0.63) and triglycerides (p=0.99). In this

Table 3 Multiple linear regression models showing independent determinants of mean carotid artery intima–media thickness (IMT) in 87 type 2 diabetic patients

	B	p	Partial r	IMT effect
Constant	−0.10	0.58	–	–
Age (years)	9.24×10^{-3}	<0.001	0.39	8.18×10^{-2}
Sex (men vs women)	11.6×10^{-2}	0.001	0.25	11.6×10^{-2}
Pulse pressure (mmHg)	3.11×10^{-3}	0.003	0.30	5.13×10^{-2}
Plasma PLTP activity (AU)	3.03×10^{-3}	0.03	0.23	5.00×10^{-2}
HDL cholesterol (mmol/l)	-9.45×10^{-2}	0.03	−0.24	-3.37×10^{-2}

B Regression coefficient or constant
IMT effect: in mm per 1 SD of the mean value for continuous variables or for men vs women
PLTP Phospholipid transfer protein

Table 4 Multiple linear regression models showing independent determinants of mean carotid artery intima–media thickness (IMT) in 83 control subjects

	B	*p*	Partial *r*	IMT effect
Constant	0.28	0.001	–	–
Age (years)	5.48×10^{-3}	<0.001	0.40	5.12×10^{-2}
Sex (men vs women)	5.84×10^{-2}	0.03	0.24	5.84×10^{-2}
Pulse pressure (mmHg)	3.75×10^{-3}	<0.001	0.41	5.19×10^{-2}
Plasma triglycerides (mmol/l, log-transformed)	11.7×10^{-2}	0.03	0.24	2.68×10^{-2}

B Regression coefficient or constant
IMT effect: in mm per 1 SD of the mean value for continuous variables or for men vs women

model, the contribution of plasma PLTP activity to carotid IMT was comparable to that of HDL cholesterol, as reflected by a similar IMT effect and a similar partial *r*-value (Table 3). Plasma PLTP activity was also an independent determinant of IMT (p=0.04) when plasma apo A-I (p=0.86) and apo B (p=0.72) instead of HDL cholesterol and VLDL+LDL cholesterol were included in the model (data not shown). In control subjects, IMT was determined by age, sex, pulse pressure and plasma triglycerides (multiple r=0.67, Table 4). In these subjects, there were no independent relationships of IMT with plasma PLTP activity (p=0.60), CRP (0.89), insulin (p=0.42), HDL cholesterol (p=0.82) and VLDL+LDL cholesterol (p=0.94) or with plasma apo A-I (p=0.84) and apo B (p=0.39). Figure 1 shows the IMT normalised to the mean age in each group according to plasma PLTP activity in diabetic and control subjects. Additional analyses in the combined groups demonstrated neither an interaction between plasma PLTP activity and the diabetic state with respect to IMT (p=0.44) nor a non-linear relationship of plasma PLTP activity with IMT (p>0.80, data not shown).

Discussion

The main new finding of this study is that carotid artery IMT is positively associated with plasma PLTP activity in type 2 diabetic patients. The magnitude of the effect of plasma PLTP activity on IMT was similar to that of HDL cholesterol, which was negatively related to IMT. Moreover, plasma PLTP activity was higher in type 2 diabetes, even after adjustment for waist circumference, plasma lipid levels, HDL cholesterol, triglycerides, CRP and insulin. Therefore, the present results are in agreement with the hypothesis that higher plasma PLTP activity is a determinant of greater IMT in type 2 diabetes mellitus. Our findings suggest that plasma PLTP activity could play a role in the increased cardiovascular risk observed in this disease.

Ultrasonographically measured carotid artery IMT is an accepted marker for subclinical atherosclerosis [20], and is a predictor of coronary artery disease and stroke both in non-diabetic and in diabetic populations [25–28]. As expected, the patients with type 2 diabetes included in the present study had a greater IMT than non-diabetic control subjects [29, 30]. The magnitude of the difference in IMT between diabetic patients and control subjects was similar to that in previous large-scale studies in which IMT was measured by a comparable method [31, 32]. Male sex and age are important determinants of IMT, as unequivocally shown in the ARIC study [23] and confirmed in this report. Systemic haemodynamic factors also contribute to enhanced intima-media thickening [33]. Blood pressure is an important modifiable risk factor for carotid artery IMT [34]. In our study, pulse pressure, which was higher in diabetic patients, was the strongest single haemodynamic variable associated with IMT. Pulse pressure is known to reflect arterial stiffness [35], which is higher in diabetic patients [36]. In the present study, of the conventional lipoprotein parameters, HDL cholesterol and plasma triglyceride levels were determinants of IMT in the diabetic patients and control subjects, respectively. While the relationships of these two lipid parameters with IMT have been documented previously [37, 38], the effects of VLDL+LDL cholesterol and plasma apo B levels on IMT did not reach significance in the diabetic or control subjects in this study. The use of lipid-lowering drugs was an exclusion criterion for participation in our study. Furthermore, plasma lipid cut-off values for lipid-lowering intervention are more stringent for diabetic than for non-diabetic subjects. This probably resulted in the selection of diabetic participants with relatively low total cholesterol levels, and may explain why VLDL+LDL cholesterol and apo B were not higher in the diabetic patients in the present study. This selection could also be responsible for the lack of a significant relationship of IMT with these lipid variables.

In the interpretation of the positive effect of plasma PLTP activity on IMT in type 2 diabetes, it is important that this relationship was not explained by associations of PLTP with HDL cholesterol and plasma triglycerides, or by relationships of plasma PLTP activity with CRP activity and insulin. Thus, it is unlikely that this relationship of plasma PLTP activity with IMT reflects an association with low-grade inflammation or insulin resistance. In comparison, the only data available so far have demonstrated that the effect of high plasma PLTP activity on cardiovascular disease is still present after controlling for lipid levels [13]. In non-diabetic control subjects, variation in IMT appeared to be less pronounced, compared with diabetic patients, and we were unable to demonstrate a significant independent

effect of plasma PLTP activity on IMT. Further analyses showed neither an interaction between plasma PLTP activity and diabetes mellitus with respect to IMT, nor a non-linear relationship of PLTP with IMT. Therefore, the absence of a significant relationship of IMT with plasma PLTP activity in control subjects is unlikely to be due to a diabetes-specific effect or to the presence of a threshold level of plasma PLTP activity on IMT. The lack of such a relationship could be due to insufficient power.

Among other possibilities, the observed relationship of plasma PLTP activity with carotid artery IMT may be attributable to a change in the quality of LDL. Patients with type 2 diabetes have small, dense LDL particles, which are especially prone to oxidative modification, and these particles contribute to the progression of coronary artery disease [39]. Elevated plasma PLTP activity has been shown to be an independent determinant of small, dense LDL in type 2 diabetes mellitus [40]. Increased plasma PLTP activity in diabetic patients may cause a redistribution of plasma vitamin E, resulting in lowering of the vitamin E content of apo B-containing lipoproteins [41]. The elevated plasma levels of oxidised LDL in type 2 diabetes show a negative correlation with the ratio of α-tocopherol to lipid in LDL [41]. Thus, increased sensitivity of LDL to oxidation may be an important mechanistic link between elevated plasma PLTP activity and increased IMT.

Finally, it is noteworthy that our study demonstrates that the PLTP-raising effect of the diabetic state remains after adjustment for waist circumference, plasma lipids, CRP and plasma insulin. Possible mechanisms responsible for the independent effect of the diabetic state in itself on plasma PLTP activity include impaired lowering by insulin [15]. Our findings do not support the idea that the higher PLTP activity levels in type 2 diabetes are due to an association with low-grade inflammation [19]. The influence of hyperglycaemia on circulating PLTP activity in humans is incompletely understood. PLTP release by HepG2 cells may increase in response to glucose in vitro [42]. In humans, however, plasma PLTP activity decreases after short-term hyperglycaemia independently of insulin [43]. In diabetic patients, we did not observe a relationship of plasma PLTP activity with actual glycaemia, but the effect of the HbA_1c level was close to significance.

In conclusion, this study suggests that the plasma PLTP activity level may represent a marker for accelerated atherosclerosis, particularly in patients with type 2 diabetes. Thus, measurement of plasma PLTP activity could be useful for the stratification of cardiovascular risk.

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