Dear Sir,

It has been clearly demonstrated that plasminogen activator inhibitor type 1 (PAI-1), the major regulator of fibrinolysis, is an independent cardiovascular risk factor. Increased PAI-1 plasma levels have been found to be associated with arterial thrombotic disease, myocardial infarction, cerebrovascular disease and peripheral vascular disease. Several studies have investigated the mechanisms that regulate PAI-1 plasma levels including components of the insulin resistance syndrome and PAI-1 promoter 4G/5G polymorphism which appears to affect both PAI-1 plasma levels and the incidence of cardiovascular disease (1). In particular, 4G allele has been found to be associated with enhanced gene expression and vascular complications in arteriosclerosis and diabetes mellitus. A genotype-dependent interaction has been demonstrated between PAI-1 levels and triglycerides in diabetic subjects (2). However, the role of age is still poorly defined and contradictory findings have been published on this matter (3). Thus, a major aim of the present investigation was to assess whether PAI-1 plasma level is age-dependent and is influenced by 4G/5G polymorphism. The sample population consisted of 238 subjects (84 men and 154 women; age range 20-83 years; mean age 50.2±13.3 years, mean BMI 28.2±5.6 Kg/m²) who volunteered for this study when attending our hospital for a routine medical evaluation. Eighty-four of them (35.5%) had type 2 diabetes, 72 (30.3%) had hypertension and 72 (30.3%) were current smokers. The enrolled subjects were free from any drug known to interfere with the fibrinolytic system such as statins, aspirin, ACE inhibitors and thiazides and without any clinical history of arteriosclerosis and diabetes mellitus. A genotype-dependent interaction has been demonstrated between PAI-1 levels and triglycerides in diabetic subjects (2). However, the role of age is still poorly defined and contradictory findings have been published on this matter (3). Thus, a major aim of the present investigation was to assess whether PAI-1 plasma level is age-dependent and is influenced by 4G/5G polymorphism. The sample population consisted of 238 subjects (84 men and 154 women; age range 20-83 years; mean age 50.2±13.3 years, mean BMI 28.2±5.6 Kg/m²) who volunteered for this study when attending our hospital for a routine medical evaluation. Eighty-four of them (35.5%) had type 2 diabetes, 72 (30.3%) had hypertension and 72 (30.3%) were current smokers. The enrolled subjects were free from any drug known to interfere with the fibrinolytic system such as statins, aspirin, ACE inhibitors and thiazides and without any clinical history of ischaemic heart disease (myocardial infarction, angina pectoris), stroke and peripheral vascular diseases. Hypertension was defined as SBP >140 and/or DBP >90 mm Hg, taken when the subject was seated on at least three different occasions. Subjects with either a positive history for diabetes mellitus or a fasting blood glucose level >7 mM after confirmation on repeat testing, were considered diabetic. Insulin sensitivity was assessed by the K index of the insulin tolerance test (KITT). Moreover, all subjects were Caucasians, consumed Mediterranean diet and none of them had an alcohol abuse history. All subjects gave informed consent, and the study was approved by the ethical committee of INRCA Hospital, Ancona. Enrolled patients were drawn at the INRCA Hospital. Overnight fasting venous blood samples were collected in resting state, between 8 and 9 a.m., to overcome the diurnal variation of PAI-1 and were immediately processed. After 10 min of centrifugation (2500 g) at 4°C, plasma was rapidly pipetted off and stored at –80°C.

The PAI-1 promoter 4G/5G genotype was analyzed using the allele-specific oligonucleotide melting technique described by Dawson, with some modifications as previously described (4). An immunoenzymatic method for PAI-1 antigen determination (Tintelize PAI-1, Biopool, Sweden) was used. This method assesses the whole PAI-1 plasma content as it is able to detect active and latent forms of PAI-1 as well as tissue plasminogen activator tPA/PAI-1 and urokinase plasminogen activator/PAI-1 complexes (5). Intra and inter-assay coefficients of variation of PAI-1 antigen were 3.6% and 4.2%, respectively. Blood concentrations of total cholesterol, HDL cholesterol, triglycerides, fasting glucose and glycosylated haemoglobin were measured by standard procedures. All statistical analyses were performed according to the SPSS statistical package. Triglycerides, KITT and PAI-1 antigen were logarithmically transformed to allow the use of parametric tests. Differences between genotypes were evaluated by analysis of variance (ANOVA) and χ² for continuous and discrete variables, respectively. The non parametric analyses (Kruskal Wallis test) were performed to confirm the results of parametric analyses for not normally distributed variables. Allele frequencies and the Hardy-Weinberg equilibrium were analyzed by χ² test. To evaluate the independent association between age and other variables including PAI-1 antigen, Pearson’s correlations and multiple regression analyses, adjusted for clinical and metabolic covariates, were performed with PAI-1 as dependent variable. These analyses were stratified by genotype. Probability values less than 0.05 were considered statistically significant. As far as PAI-1 plasma levels are concerned, no interaction between age and sex was found (F= 1.71; df=3; p=0.17). 4G/4G, 4G/5G and 5G/5G genotype were present in 57, 113 and 68 subjects, respectively. The genotype frequencies were in Hardy-Weinberg equilibrium and the allele frequencies were 47.7% for 4G and 52.3% for 5G. No significant differences were detected in PAI-1 antigen levels in the three genotypes (4G/4G: 15.6 x± 2.2 ng/ml; 4G/5G: 16.7 x± 2.1 ng/ml; 5G/5G: 17.1 x± 1.9 ng/ml, p=0.814). Neither clinical (diabetes mellitus, hypertension) nor biochemical (fasting glucose, glycosylated haemoglobin, insulin sensitivity, triglycerides, total cholesterol and HDL cholesterol) nor demographic (age, gender) characteristics differed among genotypes when

**Plasminogen activator inhibitor-1 plasma level increases with age in subjects with the 4G allele at position -675 in the promoter region**

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Received March 8, 2004
Accepted after revision September 3, 2004

Financial support:
This work has been supported in part by a grant of the Ministry of Health (ICS 030.6/RFO-49).

**Thromb Haemost 2004; 92: 1164-5**
parametric and non-parametric tests were used (data not shown). The relationships between age and the considered determinants of PAI-1 were evaluated in the whole sample. Significant correlations were found between age and fasting glucose ($r=0.36$, $p<0.01$), glycosylated haemoglobin ($r=0.28$, $p<0.01$), insulin sensitivity ($r=-0.20$, $p<0.01$), total cholesterol ($r=0.22$, $p<0.01$) and PAI-1 antigen ($r=0.16$, $p=0.03$). Stratifying by genotype, significant associations were found between age and PAI-1 antigen in 4G/4G ($r=0.38$, $p<0.01$) and 4G/5G groups ($r=0.26$, $p<0.01$). No correlation was found in 5G/5G subjects. Multiple regression analyses, adjusting for potential confounders (Fig. 1), confirmed a significant positive correlation between PAI-1 antigen and age in the whole group (partial $r=0.16$; $p=0.029$, $R^2=17.1$%), in 4G/4G and in 4G/5G subjects (Fig. 1). The most important findings of this paper are that plasma levels of PAI-1 increase with age and that this phenomenon is related to the 4G/5G polymorphism at position -675 in the promoter region of the PAI-1 gene. The statistical analysis highlights the presence of a significant relationship between PAI-1 and age in the population we recruited, characterized by a relatively high prevalence of obesity, hypertension and diabetes. Moreover, a genetic component is apparently present as 4G/4G and 4G/5G patients present a significant relationship between PAI-1 and age, after correcting for the effects of variables that are known to influence PAI-1 levels. Thus, ageing appears to be an independent risk factor for increased PAI-1 levels, and this phenomenon is evident in subjects with 4G allele, in accordance with data showing that the effect of several polymorphisms are age-dependent (6, 7). On the whole, our data can help to identify a subgroup of people with an increasing risk of CVD with age.

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References

Figure 1: Scatter plots between ln PAI-1 antigen and age by the three genotype groups. Partial correlation coefficients (partial $r$), $p$ value and $R^2$ refer to multiple regression analyses with PAI-1 antigen as dependent variable. Independent variables included in the models were: age, gender, BMI, smoking status, diabetes mellitus, hypertension, triglycerides, insulin sensitivity, total cholesterol, HDL cholesterol, fasting glucose. The natural logarithm of PAI-1 antigen, triglycerides and KIT was used.