

# Urokinase-type plasminogen activator system predicts risk of cardiovascular events in patients with angina pectoris: results of the ECAPTURE study\*

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In most Westernized societies cardiovascular diseases are the leading cause of death over the age of 45 years and one-quarter of these deaths occur in men below the age of 65 years. The haemostasis system has been identified as an important system in cardiovascular disease (CVD). The European Concerted Action on Prevention from Thrombosis by Urokinase Enhancement (ECAPTURE) has focused on the contribution of the urokinase system to CVD. In 2298 patients with angina pectoris the relationship between plasma levels of single-chain urokinase (scu-PA), urokinase antigen (u-PA) and u-PA-inhibitor complex and the risk of cardiovascular events ( $n=84$ ) during a 2 year follow-up period was studied. Plasma levels of total u-PA and u-PA-inhibitor complex predicted the risk of cardiovascular events, the adjusted relative risks of the highest quintile versus the lowest were 2.71 [95% confidence interval (CI), 1.34–5.48] and 2.34 (95% CI, 1.08–5.11), respectively. These results suggest that the urokinase system plays a role in cardiovascular disease. *Blood Coagul Fibrinolysis* 12:453–458 © 2001 Lippincott Williams & Wilkins.

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## Introduction

The risk of cardiovascular disease (CVD) is traditionally estimated by the total plasma cholesterol concentration, blood pressure, presence of diabetes, family history, gender, age and smoking habits. Recently, it has also been shown that the plasma concentration of factors of the haemostatic system, and in particular factors of the fibrinolytic system

(plasminogen activator inhibitor type-1, tissue-type plasminogen activator (t-PA) and D-Dimer) are determinants of the risk of cardiovascular disease [1–4].

Fibrinolysis is, next to platelet aggregation and coagulation, an important mechanism included in the process to secure blood flow. Three pathways

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\*According to matters agreed upon at the meeting of the ECAT Angina Pectoris Study on 8 May 1992 in Leiden, The Netherlands, that data of patients and of patients' blood samples could be used for special fibrinolysis assays (later incorporated in ECAPTURE). ECAT centres where the patients were enrolled in the study are listed in the Appendix.

have been identified in the fibrinolytic system [5]. Firstly, the extrinsic system, including the activation of plasminogen by tissue-type plasminogen activator, secondly, the factor XII-dependent pathway and finally, the urokinase-like system. The first two pathways have already been studied in relation to CVD [1–4,6,7], but only limited information is available for the latter system.

The key enzyme in the third pathway is urokinase-type plasminogen activator (u-PA). This enzyme occurs in three forms in the blood circulation: the inactive zymogen single-chain u-PA (scu-PA) (average reference value 2.2 ng/ml), the active enzyme two-chain u-PA (tcu-PA) (average reference value < 0.1 ng/ml) and u-PA-inhibitor complex, the inactive complex of tcu-PA with inhibitors (average reference value 1.1 ng/ml) (Dooijewaard G, unpublished results 1996).

The zymogen scu-PA is interesting as a fibrinolytic risk factor for CVD, because in itself it is secured from the effects of inhibitors and thereby readily available throughout the bloodstream [8,9]. Furthermore, moderate increases of scu-PA will have a proportional effect on the rate of local generation of tcu-PA (active form of u-PA), because the blood level of scu-PA is far below the  $K_m$  for activation by plasmin. Thus, increased circulatory scu-PA levels could provide for a potential amplification of the fibrinolysis process and act as a second defence mechanism against thrombosis, and decrease the risk of CVD [7,10]. In addition, uPA antigen levels have gained interest in cardiovascular disease, as their plasma levels were increased in patients with un-

stable angina [11] and predicted the risk of restenosis [12].

Within the framework of European Concerted Action on Prevention of Thrombosis by Urokinase Enhancement (ECAPTURE) (organization of study is given in the Appendix) we performed a substudy on the relationship of plasma concentrations of total u-PA, scu-PA and u-PA inhibitor complex with the severity of CVD and the risk of future cardiovascular events in patients with angina pectoris [4].

## Patients and methods

### *Patients with angina pectoris*

The population that was studied in the European Concerted Action on Thrombosis and Disability (ECAT) angina pectoris study has been described in detail elsewhere [4]. Briefly, 3043 patients from 18 European centres, who underwent coronary angiography because of suspected coronary artery disease were included in the study between October 1984 and June 1987. Patients were evaluated annually for 2 years, and information on deaths, coronary events, hospital admissions and current medication was obtained at each follow-up contact. On admission blood was collected in a standardized way [13]. For the measurement of u-PA, plasma from 2298 patients (84 cases and 2214 controls) from 14 centres was available for analysis. Patients with other major events (e.g. death from other causes, cancer) ( $n = 114$ ) were excluded from this analysis.

Patient characteristics are given in Table 1.

**Table 1.** Baseline characteristics of patients with angina pectoris in the ECAT study population

	Controls	Cases	<i>P</i>
Number of patients	2214 <sup>a</sup>	84	
Mean age (years)[mean (SD)]	55.7 (8.6)	58.1 (9.8)	0.01
Men [ <i>n</i> (%)]	1895 (86%)	75 (89%)	0.4
Smokers [ <i>n</i> (%)]	379 (17%)	17 (20%)	0.8
Diabetes [ <i>n</i> (%)]	2430 (10%)	15 (18%)	0.045
Hypertension [ <i>n</i> (%)]	722 (33%)	23 (27%)	0.2
History of myocardial infarction [ <i>n</i> (%)]	980 (44%)	57 (68%)	< 0.01
Number of occluded vessels			
none	524 (24%)	4 (5%)	< 0.01
one	590 (27%)	13 (16%)	
two	536 (24%)	27 (32%)	
three or four	558 (25%)	39 (47%)	
Ejection fraction [% (SD)]	61.1 (13.7)	55.6 (15.7)	< 0.01
Total cholesterol [mg/dl (SD)]	201 (39)	208 (33)	0.08

<sup>a</sup>Samples available for characterization of the variables of the u-PA system.

### Angiography and clinical end points

The angiographic results were expressed as the number of vessels (0 to 4) with stenosis of at least 50% of the vessel diameter or occlusion. The primary end points of the study were fatal or non-fatal myocardial infarction, and sudden death from coronary causes. Excluded from the analysis were patients with events that occurred within 72 h of surgery (usually coronary artery bypass surgery) or angioplasty, or patients with unconfirmed myocardial infarction or death from other cardiac causes, other causes or uncertain causes.

### Assay methods

**Total u-PA related antigen.** This was measured using an enzyme-linked immunosorbent assay (ELISA) as described [14]. Briefly, for the total u-PA ELISA 96-well microtitre plates were coated overnight with rabbit anti-u-PA IgG. The plates were then subsequently incubated with the plasma sample, goat anti-u-PA IgG and rabbit-anti(goat IgG) IgG conjugated with alkaline phosphatase and coloured with *p*-nitrophenyl phosphate in diethanolamine buffer. The absorbance at 405 nm was used to calculate the total u-PA concentration relative to a plasma pool calibrated against the u-PA international standard (87/894, NIBSC, Potters Bar, UK). This assay measures the u-PA antigen irrespective of its molecular form, i.e. the inactive proenzyme scu-PA, u-PA and u-PA-PAI complex [14]. In an apparently healthy reference group ( $n = 50$ ) we measured a concentration of 3.4 ng/ml (SD, 1.8).

**Scu-PA (u-PA activity).** This was measured using a biological immunoassay (BIA) as described previously [15]. Briefly, u-PA, irrespective of its molecular form, is singled out by the same rabbit polyclonal anti-u-PA IgG immobilized on microtitre plates used in the u-PA ELISA. In the next step the scu-PA is activated by incubation with human plasmin and in the final step the plasminogen activator activity is measured. In an apparently healthy reference group ( $n = 50$ ) we measured a concentration of 2.2 ng/ml (SD, 0.9).

**u-PA-inhibitor complex.** This has been calculated as the difference between the total u-PA concentration and the scu-PA levels [15]. In an apparently healthy reference group ( $n = 50$ ) we calculated a concentration of 1.2 ng/ml (SD, 1.2).

### Quality assessment

A control plasma in the reference range was

analysed 289 times for scu-PA using the BIA method with an average of three measurements per day (on separate plates). The within-day coefficient of variation (CV) was 11.5% and the between-day CV was 20.1%. The control plasma was analysed 262 times totally for u-PA antigen. The within-day CV was 6.1% and between-day CV was 11.6%.

### Statistical analysis

The distribution of the plasma u-PA-inhibitor complex concentration was skewed and therefore normalized by logarithmic transformation before the analysis. Student's *t*-test, chi-square test or analysis of covariance, using age, sex and centre as covariates, were performed to study the relationship between CVD, patient characteristics, medication and u-PA variables. Logistic regression analysis was performed to study the relation between u-PA variables and the risk of coronary events during the 2 year follow-up period. Quintiles were derived based on the distribution in the non-event group. The analyses were performed unadjusted, with adjustment for age, sex and centre, smoking habits, body mass index (BMI), total cholesterol levels, use of diuretics and digitalis. These variables are accepted cardiovascular risk factors or were associated with the u-PA variables in univariate analysis (results not shown). Because the lowest risk was expected in the lowest quintiles of u-PA antigen and u-PA-inhibitor complex and in the highest quintile of scu-PA, these quintiles were used as reference group.

Even though the inclusion and exclusion criteria were the same for all centres, a large variation between the centres was observed in the clinical history and medication of the patients. For example, the percentage of patients with diabetes ranged from 4 to 20%, the percentage of patients with exercise angina ranged from 44 to 94% and the percentage of smokers ranged from 5 to 38%. However, there was no association between the event rate in a centre and the clinical history of the patients (coronary artery bypass graft, hypertension, angina at rest, peripheral arterial disease) or the percentage of patients that used antiplatelet drugs, Ca<sup>++</sup>-antagonists, digoxin, diuretics, heparin, oral anticoagulant therapy or  $\beta$ -blockers. We assume that the inclusion policy in the different centres was indeed comparable but that there was variation in severity of the patients coming to a certain centre (a university hospital will have more severe cases than general hospitals) and in the medication strategy. We adjusted all our analyses for centre to cover all possible differences between the centres.

The statistical analyses were performed using

SPSS for Windows, release 8.5 (SPSS Inc., Chicago, Illinois, USA).

### Results

As expected, the patients who developed a cardiovascular event had more severe disease at baseline and were more often positive for established risk factors, such as smoking and diabetes (Table 1). In patients with angina pectoris who developed myocardial infarction or sudden ischaemic death during the 2 year follow-up period the u-PA antigen and u-PA-inhibitor complex levels at baseline (inclusion level) were higher (3.83 ng/ml (SE, 0.13) and 1.73 ng/ml (SE, 0.08), respectively) than the levels of these variables in patients who did not experience an event (3.49 ng/ml (SE, 0.03) and 1.48 ng/ml (SE,

0.02), respectively, with  $P = 0.01$  and  $P < 0.001$ , respectively) (Table 2). Levels of scu-PA were not significantly different in those patient groups.

The relative risk in the highest u-PA antigen quintile relative to the lowest was 2.71 [95% confidence interval (CI), 1.34–5.48], for scu-PA the relative risk for the lowest quintile was 0.57 (95% CI, 0.30–1.11) and for u-PA-inhibitor complex the relative risk in the highest quintile was 2.34 (95% CI, 1.08–5.11), after adjustment for centre, age, sex, BMI, use of digitalis and diuretics, smoking and total cholesterol levels (Table 3 and Fig. 1).

### Discussion

The main finding of this study is the fact that the plasma concentrations of total u-PA and u-PA-inhibitor complex at baseline are associated with the risk of a future coronary event in patients with angina pectoris. The results of this large European multicentre study indicate that the plasma levels of uPA antigen and of u-PA-inhibitor complex are novel and strong risk indicators.

The u-PA antigen has been shown to be present in the atherosclerotic plaque [16] and there u-PA contributes to various processes in the pathogenesis of cardiovascular disease [17] such as the stimulation of cellular migration and the conversion of plasminogen to plasmin, which results in degradation of matrix components and thus affects plaque stability [18]. Furthermore, in patients with stable coronary artery disease, the levels of total u-PA antigen were

**Table 2.** Relation between variables of the urokinase system and the development of ischaemic events

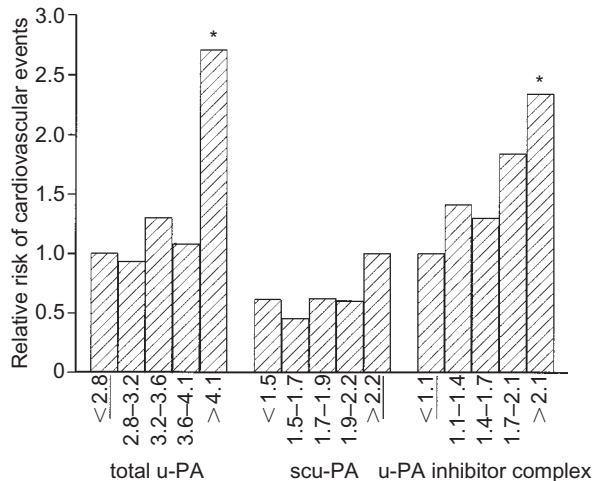
	Group with events ( <i>n</i> = 84)	Event-free group ( <i>n</i> = 2214)	<i>P</i> value ( <i>t</i> -test)
u-PA antigen	3.83 (0.13)	3.49 (0.03)	0.01
scu-PA	1.93 (0.05)	1.87 (0.01)	0.2
u-PA-inhibitor complex <sup>a</sup>	1.73 (0.08)	1.48 (0.02)	< 0.01

Values are mean (SE) in ng/ml, after adjustment for age, sex and centre. <sup>a</sup>Data logarithmically transformed before analysis. u-PA, urokinase antigen; scu-PA, single-chain urokinase.

**Table 3.** Relative risk of coronary events (95% confidence interval) according to the concentrations of factors of the urokinase system

	Quintile				
	1	2	3	4	5
Not adjusted					
u-PA antigen	1	0.92 (0.44, 1.94)	1.27 (0.62, 2.56)	0.97 (0.45, 2.07)	2.25 (1.18, 4.28)
scu-Pa	0.95 (0.52, 1.75)	0.53 (0.24, 1.16)	0.75 (0.36, 1.54)	0.74 (0.37, 1.47)	1
u-PA-inhibitor complex	1	1.45 (0.69, 3.14)	1.37 (0.62, 3.04)	1.98 (0.97, 4.07)	2.35 (1.15, 4.79)
Adjusted (for centre, age, sex, smoking, body mass index, total cholesterol, use of digitalis and diuretics)					
u-PA antigen	1	0.93 (0.44, 1.98)	1.30 (0.63, 2.70)	1.08 (0.49, 2.38)	2.71 (1.34, 5.48)
scu-PA	0.61 (0.30, 1.26)	0.45 (0.20, 1.02)	0.62 (0.29, 1.32)	0.60 (0.29, 1.23)	1
u-PA-inhibitor complex	1	1.41 (0.66, 3.02)	1.30 (0.57, 2.94)	1.84 (0.86, 3.94)	2.34 (1.08, 5.11)

Because the lowest risk was expected in the lowest quintiles of u-PA antigen and u-PA-inhibitor complex and in the highest quintile of scu-PA, these quintiles were used as reference group.



**Figure 1.** Risk of cardiovascular events in patients with angina pectoris relative to their quintiles of plasma concentrations of urokinase antigen (u-PA), single-chain urokinase (scu-PA) and u-PA-inhibitor complex. The reference quintile is the lowest quintile for total u-PA and u-PA-inhibitor complex and the highest quintile for scu-PA (reference quintile is underlined). \* $P < 0.05$ .

higher than those in healthy age-matched volunteers [19], supporting the idea that an impaired fibrinolytic capacity contributes to development and stability of atherosclerotic plaques. Furthermore experimental models of rat carotid artery balloon injury have demonstrated that smooth muscle cells and endothelial cells express u-PA in the first week after injury, coinciding with migration and proliferation of smooth muscle cells and the development of a neointima [20].

The complex of u-PA with inhibitors correlated very well with the risk of coronary events in the angina pectoris patients, but it is not yet clear what the composition of this complex is. A candidate was the complex of u-PA with protein C-inhibitor, but the plasma levels of this complex were comparable in patients with acute myocardial infarction and healthy volunteers (Binder BR, unpublished results, 1998).

In this study, the scu-PA and total u-PA antigen levels were only determined in one plasma sample. With multiple samples the predictive value would have been estimated more accurately, because the biological variation of the scu-PA and total u-PA concentrations were 59 and 52%, respectively, of the total variance in plasma from nine healthy individuals from whom blood was collected once a year during a 6 year period (Dooijewaard G, unpublished results, 1998).

Some correlations were observed between medica-

tion of the patients and scu-PA and total u-PA antigen levels (vide supra). Notably, patients using diuretics and digitalis have higher levels of u-PA antigen and uPA-inhibitor complex. The mechanism underlying these associations is not clear.

In conclusion, in patients with angina pectoris and at risk of acute ischaemic heart disease, the plasma levels of total u-PA and u-PA-inhibitor complex predict the risk of cardiovascular events. Further characterization of the uPA-inhibitor complex is expected to contribute significantly to the understanding of the pathology and the prevention of CVD.

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## Appendix

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