Soluble urokinase plasminogen activator receptor and incidence of venous thromboembolism

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Summary
Raised plasma levels of the soluble urokinase plasminogen activator receptor (suPAR) have been associated with increased incidence of cardiovascular diseases. Whether suPAR is associated with venous thromboembolism (VTE) is largely unknown. The purpose of the present study was to investigate the relationship between suPAR and incidence of VTE in a cohort study. suPAR was measured in 5,203 subjects (aged 46–68 years, 58 % women) from the general population, who participated in the Malmö Diet and Cancer (MDC) study between 1991 and 1994. Incident cases of VTE were identified from the Swedish patient register during a mean follow-up of 15.7 years. Of 5,203 subjects with measurements of suPAR, 239 had VTE during follow-up (127 venous thrombosis, 86 lung embolism, 26 both). Incidence of VTE was significantly higher in subjects with suPAR levels in the top quartile. Adjusted for age and sex, the HR (4⁰ vs 1⁰ quartile) was 1.74 (95 %CI: 1.2–2.6, p for trend=0.003). After adjustments for risk factors, the HR was 1.66 (95 %CI: 1.1–2.5, p for trend=0.016). High level of suPAR was a risk indicator for incidence of VTE in this population-based cohort study. The causal relationships between suPAR and VTE remain to be explored.

Keywords
Epidemiological studies, venous thrombosis, pulmonary embolism, plasminogen activators

Introduction
Urokinase plasminogen activator receptor (uPAR) is a glycosylphosphatidylinositol (GPI)-anchored protein which binds with high affinity to urokinase plasminogen activator (uPA), an important activator of plasminogen. uPAR is expressed on endothelial cells, activated neutrophils and monocytes (1–4) and is believed to play a role in fibrinolysis, angiogenesis, cell migration and matrix degradation. During acute and chronic inflammation uPAR is cleaved from the cell surface and released into the circulation forming soluble uPAR (suPAR) (5–7). The regulation and formation of suPAR is incompletely known, but it is believed that suPAR negatively regulates the proteolytic activity of uPA (8). suPAR has emerged as a novel inflammatory biomarker (7) and recent studies have linked suPAR to increased risk of various cardiovascular diseases and death (9–12).

Even though suPAR is closely linked to the fibrinolytic system, its role in venous thromboembolism (VTE) is unclear. A study of patients with paroxysmal nocturnal haemoglobinuria reported correlations between suPAR and decreased plasmin generation (13). However, whether suPAR is associated with increased incidence of VTE is largely unknown. Previous studies from the Malmö Diet and Cancer cohort have shown that suPAR is associated with increased incidence of cardiovascular disease, heart failure as well as increased occurrence of carotid plaque (9, 10, 12). The present paper sought to study whether raised plasma concentrations of suPAR is associated with increased risk of VTE, and whether this association is independent of common major risk factors for VTE.

Materials and methods
Study population
The Malmö Diet and Cancer study (MDC) is a prospective cohort study from the city of Malmö in southern Sweden. A total of 28,449 participants (11,246 men, born 1923–1945; 17,203 women, born 1923–1950) attended the baseline examination between March 1991 and September 1996. Between October 1991 and February 1994, a randomly selected subgroup was invited to an extended study of the epidemiology of cardiovascular diseases (9, 10, 14). A total of 6,103 subjects accepted and 5,540 of them donated fasting blood samples at a separate visit. suPAR was analysed in 5,378 subjects. After exclusion of subjects with a history of VTE...
and individuals with a diagnosis of cancer within the last five years, the final study population consisted of 5,203 subjects.

All participants provided written informed consent, and the study was approved by the ethics committee at Lund University, Lund, Sweden (LU 51/90).

Measurements and definitions

Information about use of medication, medical history and smoking habits were obtained from a self-administered questionnaire (9, 10, 14). Weight and height were measured to the nearest 0.1 kg and 0.5 cm, respectively, with subjects wearing light clothing and no shoes. Body mass index (BMI) was calculated as weight in kilograms divided by height in meters squared (kg/m²). Blood pressure was measured using a mercury-column sphygmomanometer after 10 minutes (min) of rest in the supine position. Diabetes was defined as fasting whole blood glucose level greater than 109 mg/dl (i.e. 6.0 mmol/l), self-reported physician’s diagnosis of diabetes or use of anti-diabetic medications (15).

History of atrial fibrillation was defined as a hospital diagnosis of atrial fibrillation and history of cardiovascular disease (CVD) was defined as a diagnosis of acute myocardial infarction or stroke. The Swedish Inpatient Register (16) was used for case-retrieval.

Subjects were categorized into current smokers (i.e. those who smoked regularly or occasionally) or non-smokers (i.e. former smokers and never smokers). Subjects (n=126, 2.4 %) with missing information about smoking were coded in a dummy variable and used as a separate category in the multivariate analysis.

suPAR was analysed from frozen plasma samples. The samples were taken at the baseline examination in 1991–1994 and stored in -80°C until analysis in 2012. A commercial ELISA suPARnostic® kit (Virogates, Copenhagen, Denmark) was used. The inter-assay coefficient of variation (CV) was 5 % and the intra-assay CV was 3 %. suPAR has shown to have high stability in plasma samples and remains stable through several freezing and thawing cycles (17).

The assessment of hsCRP (mg/l) was performed using the Tina-quant® CRP latex high sensitivity assay (Roche Diagnostics, Basel, Switzerland) on an ADVIA® 1650 Chemistry System (Bayer Healthcare, New York, NY, USA).

DNA was extracted from peripheral blood cells. Genotyping was performed at the Broad Institute, Boston, USA, using the Illumina HumanOmniExpress Exome Bead Chip version 1.0, and the iScan system (Illumina, San Diego, CA, USA), using the Auto-call calling algorithm. The array included >240,000 exome variants, and >700,000 markers for coverage of the genome-wide variation. The Factor 5 Leiden (F5L) (rs6025) polymorphism was imputed according to 1000Genomes, build 37, using Impute 2 software (www.1000genomes.org; 18). Genetic data for imputation of the F5L mutation was available for 4,511 individuals.

Ascertainment of endpoint

All subjects were followed from the baseline examination until a first event of VTE (primary or secondary diagnosis), emigration, death or December 31, 2009, whichever came first. VTE was defined as International Classification of Diseases codes (ICD-8, used before 1987) 450 (lung embolism, LE) and 451 (deep-vein thrombosis [DVT] of the lower limbs); ICD-9 codes (used 1987–1996) 415B (LE), and 415B (DVT of the lower limbs); and ICD-10 codes (used 1997–2009) I26 (LE), and I80 (DVT of the lower limbs). Patients with superficial thrombophlebitis were excluded (ICD-9 code 451A and ICD-10 code I80.0). The Swedish inpatient and out-patients registers (16, 19) and the cause of death register were used for case retrieval. The inpatient register and cause of death register have been operating during the whole follow-up period, and the out-patient register covers hospital outpatient visits from 2001 and onwards. Validation studies from the patient register have shown high case validity of a diagnosis of VTE (95 %) (20) and for other cardiovascular disorders (21, 22). It has been shown that almost all VTE patients in the city are diagnosed with objective methods such as phlebography, ultrasound or computer tomography (23).

Statistical analysis

The subjects were categorised according to suPAR levels into sex-specific quartiles, i.e. with similar proportions of men and women in each quartile. suPAR was also log transformed and analysed as a continuous variable per 1 standard deviation of the log values. One-way analysis of variance (for continuous variables) and Pearson’s Chi² (for dichotomous variables) were used to study the distribution of risk factors across quartiles of suPAR. P for trend across quartiles of suPAR was calculated by fitting the quartiles as an ordinal variable. Cox proportional hazards regression was used to examine the association between suPAR and incidence of VTE. Time axis was time from the baseline examination until death, emigration, incident VTE or end of follow-up, whichever was first. Age and sex were included as covariates in the basic model. Secondly, we also adjusted for history of CVD (stroke or acute coronary event), atrial fibrillation, systolic blood pressure (SBP), leucocyte count, diabetes, BMI, current smoking, total cholesterol and high-density lipoprotein cholesterol (HDL). C-reactive protein (CRP) was missing for 207 (4.0 %) individuals. The effect of CRP (log transformed) was explored in a sensitivity analysis after excluding those without this information. Interaction terms were added to the full multivariate model to explore potential interactions between suPAR and other risk indicators. The fit of the proportional hazards model was checked visually by plotting the incidence rates over time and by using time dependent variables in the model.

Results

Baseline characteristics

A total of 5,203 subjects were available for the analysis. Median suPAR was 2.72 ng/ml (interquartile range [IQR]: 2.34–3.32) in men and 2.92 ng/mL (IQR: 2.49–3.49) in women. The relationships between quartiles of suPAR and other baseline characteristics are presented in Table 1. As expected, suPAR was
significantly associated with age, BMI, smoking, HDL, diabetes and blood pressure. There was no association between suPAR and the F5L mutation.

**suPAR and incidence of VTE**

During a mean follow-up of 15.7 ± 3.8 years, a total of 239 (96 men, 143 women) had VTE. Incidence of VTE was significantly higher in subjects with suPAR levels in the top quartile (Figure 1, Table 2). Adjusted for age and sex, the hazard ratio (HR) (4th vs 1st quartile) was 1.74 (confidence interval [CI]: 1.2–2.6, p for trend=0.003). After adjustments for potential risk indicators, the HR was 1.66 (CI: 1.1–2.5, p for trend: 0.016). The results were essentially unchanged after further adjustment for F5L mutation in the subgroup with genetic information (HR, 4th vs 1st quartile: 1.58; CI:1.01–2.46, p for trend= 0.03). There was no significant interaction between F5L and suPAR, with respect to incidence of VTE.

After multivariate adjustments, the relationship between suPAR was significant for women (HR, 4th vs 1st quartile: 1.9 (CI: 1.1–3.5); p for trend= 0.015), but not for men (HR: 1.3 (CI: 0.7–2.5); p for trend= 0.50). The HR in women was unchanged when current use of hormone replacement therapy was added to the model (HR: 1.9; CI:1.08–3.4, p for trend=0.018). However, there was no significant interaction between gender and suPAR with respect to incidence of VTE (p=0.13). The incidence rates in men and women are illustrated in Suppl. Figures 1 and 2 (available online at www.thrombosis-online.com).

When incidence of DVT and LE was analysed separately, suPAR was significantly associated with DVT (HR, Q4 vs Q1: 1.84 (CI:1.1–3.1); p for trend=0.011). suPAR was associated with LE in women (but not in men) after adjustment for age. There was no significant relationship with LE in the total sample after adjustments for risk factors (HR, Q4 vs Q1: 1.34 (CI: 0.72–2.5); p for trend 0.31). Incidences of LE and DVT, respectively, are illustrated in Suppl. Figures 3 and 4 (available online at www.thrombosis-online.com).

**Adjustments for CRP**

CRP was available in 4996 individuals (224 VTE events). The Spearman rank correlation between suPAR and CRP was 0.26. The HR for suPAR in this subgroup was 1.54 (4th vs 1st quartile; CI: 1.04–2.3, p for trend=0.0501) after adjustments for risk indicators. The HR was essentially unchanged (HR=1.53; CI:0.995–2.3, p for trend= 0.056) after further adjustment for CRP (log-trans-
formed). There was no relationship between CRP and incidence of VTE (HR per 1 SD increase: 1.01, CI: 0.88–1.16, p=0.86).

Discussion

suPAR is a novel marker of inflammation and raised suPAR concentrations have been associated with incidence of cardiovascular disease and death. Even though uPA and uPAR are important parts of the fibrinolytic system, the role of the soluble receptor suPAR in VTE is unclear. The present population-based study showed that raised suPAR is a risk indicator for incidence of VTE, even after adjustment for several potential confounding factors.

uPA and the cellular receptor uPAR are important components of plasminogen activation and thrombolysis (24). Besides its role in plasminogen activation, uPAR is involved in cell signalling (25), leukocyte recruitment (26), tissue modelling (27) and angiogenesis (28). The function of the receptor is regulated by cleavage of one of the three receptor domains or by releasing the soluble full length of the receptor (suPAR) (8). Hence, suPAR could compete with membrane bound forms of uPAR and thereby reduce the uPA proteolytic activity. Reduced uPA activity could be a possible mechanism for the increased incidence of VTE in subjects with high suPAR.

The relationship between suPAR and incidence of VTE reached significance for women, but not for men. This could be explained by low statistical power when men were analysed separately, and there was no significant interaction between suPAR and gender with respect to incidence of VTE. Similarly, we found significant relationships between suPAR and DVT, but no significant relationship with LE. Again, this could be a result of limited power. However, difference in fibrinolytic activity between DVT and LE has been reported (29) and it cannot be excluded that suPAR mainly could be associated with DVT.

Table 2: Incidence of VTE over a mean follow-up of 15 years, in relation to quartiles of suPAR.

<table>
<thead>
<tr>
<th>suPAR (ng/ml)</th>
<th>Q1</th>
<th>Q2</th>
<th>Q3</th>
<th>Q4</th>
<th>P, trend</th>
<th>Per 1 SD log suPAR</th>
</tr>
</thead>
<tbody>
<tr>
<td>(men/women)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;2.34/</td>
<td>2.34–2.72/</td>
<td>2.72–3.32/</td>
<td>&gt;3.32/</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;2.49</td>
<td>2.49–2.92</td>
<td>2.92–3.49</td>
<td>&gt;3.49</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N (men/women)</td>
<td>540/760</td>
<td>540/761</td>
<td>540/761</td>
<td>540/761</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DVT/PE/both, n</td>
<td>23/14/6</td>
<td>29/24/2</td>
<td>28/27/8</td>
<td>47/21/10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age and sex adjusted HR</td>
<td>1.00</td>
<td>1.18 (0.79–1.8)</td>
<td>1.29 (0.87–1.9)</td>
<td>1.74 (1.2–2.5)</td>
<td>0.003</td>
<td>1.90 (1.24–2.90)</td>
</tr>
<tr>
<td>Risk factor-adjusted HR*</td>
<td>1.00</td>
<td>1.20 (0.79–1.8)</td>
<td>1.27 (0.84–1.9)</td>
<td>1.66 (1.1–2.5)</td>
<td>0.016</td>
<td>1.76 (1.09–2.8)</td>
</tr>
</tbody>
</table>

* adjusted for age, sex, BMI, smoking, systolic blood pressure, cholesterol, HDL, leukocyte count, diabetes, history of atrial fibrillation, history of cardiovascular disease. 5,086 individuals and 233 VTE events were available for the risk factor adjusted model. DVT, venous thrombosis; PE, pulmonary embolism; VTE, venous thromboembolism (DVT or PE).
Previous studies have shown that suPAR is associated with arterial CVD and atherosclerosis in the carotid arteries (9–12). It has also been reported that VTE is associated with occurrence of arterial CVD (30). Hence, one could speculate whether the increased incidence of VTE could be related to arterial CVD rather than suPAR per se. However, the results were largely unchanged after adjustments for history of coronary events or stroke and several cardiovascular risk factors. This suggests that the relationship with VTE is independent of arterial CVD.

The endpoints were retrieved from national registers covering hospital out-patient and in-patient care in Sweden (16). A study of VTE in this register reported a case validity of 95% (20). Previous studies from Sweden reported that almost all VTE patients are diagnosed with objective methods, such as phlebography, ultrasound, or computer tomography (23, 31). However, the endpoint definition obviously does not include cases of VTE that are undetected or do not seek medical care. A limitation is that we could not distinguish proximal and distal DVT.

The F5L mutation was imputed using a whole genome scan. suPAR was not associated with the mutation, and since suPAR and factor V are involved in two different pathophysiological pathways, this was in line with our expectations. However, our analysis did not include family history or other less common genetic risk factors for VTE.

All patients with self-reported physician-treated cancer during the last five years were excluded. Some risk factors for VTE were unavailable, which is a limitation of the study. We lack information about major surgery or trauma during the follow-up period. However, since plasma samples for suPAR were collected several years before the VTE events, it is unlikely that subsequent trauma or surgery could be a cause of bias in this study. It has been reported that hyperthyroidism is associated with increased risk of VTE (32). We had no information about plasma levels of thyroid hormones, and it is unclear if suPAR is related to thyroid function. We cannot rule out that residual confounding is possible by various comorbidities or concomitant drugs not adjusted for in the analysis.

It is concluded that high suPAR concentration is associated with increased incidence of VTE in this prospective cohort study from the general population. The causal relationships between suPAR and VTE remain to be explored.

Conflicts of interest
None declared.

References