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Abstract

Background: Soluble urokinase plasminogen activator receptor (suPAR) is a highly sensitive marker that reflects increased inflammation and is positively correlated with pro-inflammatory biomarkers. The aim of this study was to explore the relationship between suPAR, cardiovascular disease (CVD) risk factors and incidence of CVD

Methods: suPAR was assessed in a random sample of participants (N=569), aged 63-68 years (mean age 65.5), from the Malmö Diet and Cancer Study (MDCS) cardiovascular cohort. Baseline examination was conducted between 1991 and 1994. suPAR in blood was analyzed using a commercially available assay (suPARnostic). Cox regression analysis was used to investigate the incidence of CVD (coronary events or ischemic stroke), in relation to sex-specific tertiles of suPAR.

Results: Significantly higher plasma levels of suPAR was found in women, smokers, diabetics and older subjects. suPAR was significantly positively correlated with markers of systemic inflammation (i.e. high sensitive C-reactive protein (hsCRP) and white blood cells (WBC), but not to lipoprotein-associated phospholipase A2 (Lp-PLA₂), a specific vascular inflammatory biomarker.

87 subjects had a CVD event during follow-up (mean 14.1 years). In an age/sex-adjusted model, the hazard ratio (HR) for incident CVD was 2.53 (95%CI: 1.44-4.46) for the top compared to the bottom tertile of suPAR. This association remained significant after further adjustment for smoking, low density lipoprotein (LDL), systolic blood pressure, use of anti-hypertensive medication, diabetes, hsCRP, WBC and Lp-PLA₂ (HR: 2.25; 1.07-4.72).

Conclusion: Elevated levels of suPAR are, independently of established cardiovascular risk factors, associated with an increased incidence of CVD in elderly subjects.

Soluble urokinase plasminogen activator receptor in plasma is associated with incidence of CVD. Results from the Malmö Diet and Cancer Study

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Introduction

Intensive research efforts over the past decades have increased our understanding of the pathogenesis of atherosclerosis, atherothrombosis and their clinical complications. Today atherosclerosis is known to be a chronic inflammatory process, from initial plaque formation to destabilization and rupture^{1,2}. Blood-borne cells (monocytes, macrophages, T-lymphocytes) play an important role in promoting the inflammatory processes¹, and population-based studies have investigated the relation between several inflammatory markers and cardiovascular disease (CVD)^{3,4}.

Soluble urokinase plasminogen activator receptor (suPAR) is an emerging inflammatory biomarker. This biomarker shows high stability during storage and after repeated freeze and thaw cycles, and is therefore suitable for analysis in frozen plasma samples^{5,6}. Elevated levels of suPAR have shown to be associated with worse outcome in patients with various infectious diseases and cancer⁷⁻¹⁰. Less is known about the relationship between suPAR and risk of CVD. Results from the Danish MONICA10 (MONItoring trends and determinants of Cardiovascular disease) study showed that moderately increased levels of suPAR were related to increased risk of cancer, type 2 diabetes mellitus and CVD¹¹. The relation between elevated suPAR and increased risk of CVD was stronger for the younger age-group. Another study from the same cohort concluded that suPAR was related to subclinical organ damage (i.e. high pulse wave velocity, presence of carotid plaques and urine albumin/creatinine ratio). Elevated levels of suPAR were associated with increased risk of future CVD independently of traditional risk factors and subclinical organ damage¹².

The aim with the present study was to examine the relationship between suPAR and two non-specific (CRP, leukocyte count) and one vascular (LpPLA₂) marker of inflammation, and investigate if elevated levels of suPAR are associated with increased risk of CVD in an elderly population.

Methods

Subjects

The Malmö Diet and Cancer Study (MDCS) is a prospective population-based cohort study with the purpose of exploring the effects of dietary habits on risk of cancer¹³. All men and women between 45 and 69 years old, living in the city of Malmö Sweden, were invited.

Between October 1991 and February 1994, a random sample of participants was invited to take part in the sub-study of the epidemiology of carotid artery disease¹⁴. This cardiovascular cohort consists of 6103 subjects (aged 45-68 years, 60 % women). To the present study a random sample (n=600) from those aged 63-68 years (mean age 65.5 years) was used, of whom 63 percent were women. Subjects with history of CVD (n=31, 26 % women) were excluded, thus the study population consist of 569 subjects. All participants provided written informed consent and the study was approved by the Ethical Committee at Lund University, Lund, Sweden (LU 51/90).

Baseline examination

Anthropometrics, blood sampling, blood pressure and a self-administered questionnaire (including previous and current diseases, medication and life style factors, e.g. smoking habits and physical activity) were obtained at the baseline examination and have been described in detail previously^{15, 16}. Fasting blood samples for analysis of lipids and glucose status were drawn and aliquots of plasma were stored at minus 80 degrees.

Smoking was classified as current, former- and never smoker. Diabetes was defined as self-reported physician diagnosis per questionnaire or current treatment with anti-diabetic drugs or fasting whole blood glucose equal or above 6.1 mmol/L. Blood pressure was measured once after 10 minutes rest in supine position. Hypertension was defined as current use of anti-

hypertensive treatment or a systolic or diastolic blood pressure ≥ 140 or ≥ 90 mmHg, respectively.

Blood glucose, total and high density lipoprotein cholesterol (HDL) and WBC (cells /L) was measured according to standard procedures at the Department of Clinical Chemistry, Skåne University Hospital, Malmö. Low density lipoprotein cholesterol (LDL) concentration was calculated according to Friedewalds formula. hsCRP was analysed using the Tina-quant® CRP latex assay (Roche Diagnostics, Basel, Switzerland) on an ADVIA® 1650 Chemistry System (Bayer Healthcare, NY; USA). Lp-PLA2 activity (ng/min/mmol) was determined via a radiometric high-throughput activity assay. Lp-PLA2 mass concentration (ng/mL) was measured using a commercially available enzyme-linked immuno-sorbant assay (ELISA) kit, PLAC test® (diaDexus Inc., USA) ¹⁶. Plasma drawn at baseline and stored at minus 80 degrees was used to measure plasma levels of suPAR by using the commercially ELISA suPARnostic® kit (ViroGates, Copenhagen, Denmark). The inter-assay coefficient of variation (CV) was 9.2 percent and the intra-assay CV was 2.1 percent.

Classification of events

For case retrieval the Swedish Hospital Discharge Registry ¹⁷, the Stroke register of Malmö ¹⁸, and the National Cause of Death Registry were used. The ascertainment of cases and the validity of these registries has shown to be high ^{17, 18}. All subjects were followed from the baseline examination until first occurring CVD event, death, emigration from Sweden or until December 31st 2008. A CVD event was defined as non-fatal myocardial infarction or death due to ischemic heart disease (ICD codes 410-414) or ischemic stroke (ICD code 434). The latter was verified by computer tomography, magnetic resonance imaging or autopsy as previously described ¹⁹. By definition, transient ischemic attacks (ICD-9 code 435) were not counted as CVD events.

Statistical methods

SPSS 17.0 was used for the statistical analysis. Triglyceride and hsCRP were markedly skewed and therefore log-transformed. Means (medians for skewed variables) and percentages for baseline characteristics were computed for the entire cohort and by sex. Chi-squared test were used to determine differences in baseline characteristics. To assess the correlations between suPAR and selected inflammatory markers (WBC, hsCRP and Lp-PLA₂ activity and mass) Spearman correlation coefficient was used. One-way analysis of variance (ANOVA) was used to test differences in mean value in different sub-groups. Cox regression was used to investigate the incidence of CVD in relation to sex-specific tertiles of suPAR (using the lowest tertile as referent). A basic model included age and sex; the second model also included smoking, LDL, systolic blood pressure, anti-hypertensive treatment, and diabetes and in the final model other inflammatory markers (i.e. WBC, hsCRP and Lp-PLA₂) were added. To examine whether the relationship between suPAR and incidence of CVD was independent of smoking, we also excluded smokers from the cohort and constructed new sex-specific tertiles. The area under the receiver operator characteristic (ROC) curve was tested for suPAR and hsCRP as continuous variable in relation to incident CVD. Two-tailed p-values <0.05 were considered statistically significant.

Results

Baseline characteristics for the total cohort and for men and women, respectively, are shown in Table 1. Significantly higher plasma levels were found in women (3.28 ng/ml) compared to men (2.97 ng/mL, $p < 0.001$). Median suPAR (interquartile range) was 3.13 (2.59, 3.76) for women and 2.78 (2.35, 3.49) for men. Smokers, subjects above 65 years, and CVD cases also had significant higher levels of suPAR (Table 2).

Correlation between suPAR and inflammatory markers

suPAR was highly correlated to both hsCRP and WBC ($r=0.295$ and $r=0.193$, respectively, both p -values <0.01) but there were no relation to Lp-PLA₂ activity or mass ($r=0.006$, p -value= 0.9 and $r=0.084$, p -value= 0.059 , respectively).

Baseline suPAR and incidence of CVD events

During follow-up (mean time 14.1 year) 87 CVD events occurred (47 acute coronary events, 40 ischemic strokes). Because women have significantly higher suPAR levels we constructed sex-specific tertiles of suPAR for the Cox regression analysis. suPAR was significantly associated with increased risk of CVD. In an age and sex-adjusted model the hazard ratio (HR) was 2.53 (95% confidence interval (CI) 1.44 – 4.46), for the top tertile compared to bottom tertile of suPAR. The HR was 2.53 (CI: 1.31-4.89) after smoking, total cholesterol, HDL, SBP and diabetes were taken into account. When the inflammatory markers hsCRP, WBC, Lp-PLA₂ were added, the HR was attenuated but remained statistically significant (HR; 2.25: 1.07-4.72, Table 3). The HR was 1.41 (CI: 1.03-1.94, per 1 ng/mL) when suPAR was fitted as a linear variable in a fully adjusted model. Besides suPAR, age (HR; 1.40: 1.07-1.84, per year), male sex (HR; 1.80: 1.03-3.16), antihypertensive medication (HR; 1.95: 1.08-3.52), and hsCRP (HR; 1.33: CI 1.03-1.72, per 1 mg/L), were also significantly associated with CVD in the final model.

As smoking is strongly associated with levels of suPAR, we explored if the results were consistent when non-smokers were analysed separately. There were 13.3, 13.4 and 35.2 percent smokers in suPAR tertile 1-3, respectively. When excluding smokers ($n=141$) from the cohort, the HR for a future CVD using the basic model was 2.55 (CI 1.21-5.37), comparing the first and third tertiles of suPAR.

The area under the ROC curve for suPAR and hsCRP as continuous variables was 0.54 and 0.57, respectively.

Discussion

The results from this population-based study show that elevated levels of suPAR are associated with an increased incidence of CVD even in elderly subjects. This relationship was independent of traditional risk factors and several other inflammatory markers and the results remained significant after excluding smokers from the cohort.

A study based on the Danish MONICA-cohort was the first to show association between elevated levels of suPAR and increased risk of CVD, type 2 DM, and cancer ¹¹, independently of traditional risk factors and subclinical organ damage. The strength of the association was strongest for the younger age-group (aged 41 years at baseline examination in 1993-1994) ¹¹. The present study showed a significant relation between elevated suPAR and increased risk of CVD in an older age-group (mean age 65.5 years).

Smokers have significantly higher levels of suPAR. The proportions of smokers with regard to quartile of suPAR in the Danish MONICA cohort were 25, 35, 50, and 75 percent, respectively, and corresponding figures in our study were 13.3, 13.4, and 35.2 %. One explanation for the strong relation between the suPAR and clinical outcomes could be that smokers influence the results. Our study has a low proportion of smokers (20.6%) and suPAR was significantly related to increased risk of CVD after taking smoking into account. To further explore the impact of smoking we excluded all smokers from analysis and found that suPAR was associated with CVD also in non-smokers. Thus, it is unlikely that the relationships between suPAR and CVD are completely explained by smoking.

In the present study we did not find any relation between suPAR and the vascular inflammatory marker Lp-PLA₂, which has shown to be related to CVD ²⁰ and is increased in

rupture-prone plaque compare to stable plaque²¹. In the Danish MONICA study they investigated if subjects with carotid plaque and elevated levels of suPAR were at higher risk but they could not find any relationship¹². Our data indicate that suPAR is not associated with vascular inflammation, as measured by Lp-PLA₂. This finding indicates that these two inflammatory markers reflect distinctively different mechanisms in the pathogenesis of CVD. suPAR was only moderately correlated with the non-specific inflammatory markers hsCRP and WBC which is consistent with other studies^{11,22}. In terms of ROC-statistics, suPAR showed somewhat weaker relationships with CVD than hsCRP (0.54 vs 0.57). However, the weak correlation between suPAR and other inflammatory markers suggest that suPAR represents a separate pathway and therefore could add further prognostic information.

suPAR has a role within the fibrinolytic system, but it also seems that this proteinase receptor participates in a large range of cellular responses, such as cellular adhesion, differentiation, proliferation and migration in a non-proteolytic fashion²³. Whether changes in the fibrinolytic system explains the increased risk of disease, related to suPAR, is not well known and need to be further elucidated. It is unclear whether suPAR is causally related to CVD or whether suPAR serves as a risk marker. In the present study and other studies, smoking, female sex, and age are significantly related to increased plasma levels of suPAR. However suPAR remained significantly related to increased risk of CVD, taking these factors into account.

Limitations

This study has several strengths, however also limitations. National and regional registries were used to ascertain cases of coronary heart disease and ischemic stroke. The validity from these registries has been shown to be very high^{17,18}. Although MDC is a large population-

based study, the present study is based on only a random sample of elderly subjects and not the entire cohort. In addition, the attendance rate in MDC was rather low (41%), the study participants were found healthier and at lower risk compared to non-participants^{24,25}. As a result, the observed results between suPAR and risk of CVD in the present study may be an underestimation of the true relationships.

Conclusions

In conclusion, in this prospective population-based study of elderly subjects, elevated levels of suPAR are associated with increased incidence of CVD events. This relationship was independent of traditional risk factors and selected inflammatory markers. suPAR may have clinical implications as a biomarker for identifying subjects with high risk of developing CVD.

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Conflict of interest

Prof Gunnar Engström is employed as senior epidemiologist by AstraZeneca R&D.

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Table 1. Baseline characteristics in the total cohort and by gender

Variable	Total cohort n=569	Women n=356	p-value	Men n=213
Age (years)	65.6 (1.1)	65.5 (1.08)	0.247	65.6(1.08)
Female (%)	62.6			
Smoker (%)	20.6	20.2	0.419	21.3
Cholesterol (mmol/L)	6.5 (1.22)	6.77 (1.26)	< 0.001	6.06 (1.00)
HDL (mmol/L)	1.39 (0.36)	1.47 (0.36)	< 0.001	1.24 (0.33)
LDL (mmol/L)	4.44 (1.04)	4.60 (1.07)	< 0.001	4.18 (0.94)
Triglycerides (mmol/L)	1.23 (0.93, 1.77)	1.26 (0.91, 1.78)	0.476	1.21 (0.94, 1.72)
Glucose (mmol/L)	4.9 (4.7, 5.4)	4.9 (4.6, 5.4)	0.265	5.0 (4.7, 5.6)
HbA1C (%)	5.0 (4.6, 5.3)	4.9 (4.6, 5.3)	0.231	5.0 (4.6, 5.2)
Diabetes (%)	12.7	11.2	0.119	15.0
Syst BP (mmHg)	151 (19.5)	150 (19.9)	0.290	152 (19.4)
Diast BP (mmHg)	88.5 (9.2)	87.9 (9.0)	0.041	89.5 (9.4)
Hypertension (%)	80.1	78.7	0.148	82.6
hsCRP (mg/L)	1.6 (0.8, 3.3)	1.7 (0.8, 3.3)	0.878	1.5 (0.7, 3.1)
WBC (10 ⁹ /L)	6.07 (1.53)	6.08 (1.5)	0.772	6.04 (1.6)
Lp-PLA ₂ act (ng/min/mL)	47.2 (12.8)	45.2 (12.6)	< 0.001	50.5 (12.5)
Lp-PLA ₂ mass (ng/mL)	281.7 (87.1)	269.8 (87.1)	< 0.001	301.5 (83.6)
suPAR (ng/mL)	3.17 (0.88)	3.28 (0.90)	< 0.001	2.97 (0.82)

Data are presented as mean (\pm SD), as median (inter quartile range) or as percentages.

HDL indicates high density cholesterol; LDL, low density cholesterol; Syst BP, systolic blood pressure; Diast BP, diastolic blood pressure; hsCRP, high sensitive C-reactive protein; WBC, white blood cell count;

Lp-PLA₂, lipoprotein-associated phospholipase A2 and suPAR, soluble urokinase plasminogen activator receptor
p-values for differences between men and women

Table 2. Mean levels of suPAR (\pm SD) in different sub-groups

		p-value
\leq 65 year (n=266)	3.08 \pm 0.83	
> 65 year (n=303)	3.24 \pm 0.92	0.023
Non-smokers (n=439)	3.05 \pm 0.83	
Smokers (n=116)	3.39 \pm 0.95	<0.001
No DM (n=497)	3.14 \pm 0.84	
Diabetes (n=72)	3.52 \pm 1.10	0.054
No CVD event (n=482)	3.14 \pm 0.83	
CVD (n=87)	3.34 \pm 0.91	0.015
No HT (n=113)	3.09 \pm 0.83	
HT (n=456)	3.18 \pm 0.89	0.326

suPAR, soluble urokinase plasminogen activator receptor (ng/mL)

DM, diabetes mellitus; CVD, cardiovascular disease; HT, hypertension

Table 3. Risk of CVD by sex-specific tertiles of suPAR during 14 years follow-up.

	T1	T2	T3
suPAR ng/ml	(1.5-2.8)	(2.5-3.5)	(3.2-8.6)
n (men/women)	71/119	71/118	71/119
CVD			
Events (n=87)	n=21	n=33	n=33
Model 1	reference	1.94 (1.12- 3.63)	2.53 (1.44-4.46)
Model 2	reference	2.06 (1.11-3.84)	2.53 (1.31-4.89)
Model 3	reference	1.71 (0.89-3.32)	2.25 (1.07-4.72)

Model 1: adjusted for age and sex (including 87 CVD events)

Model 2: model 1 plus adjustment for smoking, LDL, systolic blood pressure, antihypertensive medication, diabetes (70 CVD events)

Model 3: model 2 plus adjustment for hsCRP, WBC, Lp-PLA₂ activity and mass. (62 CVD events)