Serum levels of the soluble urokinase plasminogen activator receptor (suPAR) correlates with disease activity in early rheumatoid arthritis and reflects joint damage over time

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Soluble urokinase plasminogen activator receptor (suPAR) is intensively studied as a biomarker of inflammation and disease outcome in various diseases. In rheumatoid arthritis (RA), suPAR have shown an association with inflammation and swollen joints, but data on suPAR in relation to early disease course and disease progression are lacking. This study investigates the potential of suPAR to predict or reflect disease outcome in early RA. Serum suPAR was measured by enzyme-linked immunosorbent assay at disease onset and after 3 and 36 months in 252 patients from a Swedish prospective observational early RA cohort. Levels and changes of suPAR were analyzed in relation to the 28-joint disease activity score (DAS28) and joint damage according to the Larsen score at inclusion and during follow-up. 100 healthy blood donors served as controls. Circulating levels of suPAR were higher in RA patients at all time points as compared to healthy controls. Baseline suPAR was significantly associated with baseline disease activity whereas suPAR levels at 36 months were associated with joint damage at 36 months. No predictive value of suPAR levels or changes in suPAR levels over time were found. In conclusion, suPAR levels associate with disease activity in early untreated RA and reflects joint damage at later stages. Increased suPAR in established RA could indicate patients in need of frequent monitoring of joint status, irrespective of disease activity. In the view of suPAR as a rapidly emerging biomarker, it is important to be aware of its ability to reflect both inflammation and subsequent damage. (Translational Research 2021; 232:142–149)

Abbreviations: ACPA = anti–citrullinated protein antibodies; ACR87 = American College of Rheumatology 1987; CCP = citrullinated cyclic peptide; CRP = C-reactive protein; CV = coefficient of variation; DAS28 = 28-joint disease activity score; ELISA = enzyme-linked immunosorbent assay; ESR = erythrocyte sedimentation rate; RF = rheumatoid factor; RA = rheumatoid arthritis; suPAR = soluble urokinase plasminogen activator receptor; uPA = urokinase plasminogen activator

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AT A GLANCE COMMENTARY
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Background
Soluble urokinase plasminogen activator receptor (suPAR) is intensively studied as a biomarker of inflammation and outcome in various diseases. Herein, suPAR in serum was evaluated over time in recent-onset rheumatoid arthritis (RA).

Translational Significance
suPAR correlated with disease activity at baseline, whereas follow-up levels associated with structural joint damage. Thus, increased suPAR in patients with established RA may reflect joint destruction. Due to the emerging interest in suPAR as a prognostic biomarker, it is important to acknowledge that suPAR may reflect different aspects of disease depending on disease duration.

INTRODUCTION
The membrane-bound urokinase plasminogen activator receptor (uPAR; CD87) is a multi-ligand receptor encoded by the PLAUR gene and expressed on endothelial cells, smooth muscle cells, fibroblasts and immune cells such as neutrophils and monocytes.1 The receptor and its soluble form suPAR, is involved in processes like proteolysis, tissue remodeling, cell migration and inflammation via interaction with its ligand urokinase plasminogen activator (uPA; also known as urokinase), and lateral interactions with other receptors such as integrins, vitronectin and formyl peptide receptors.1,3

Increased uPAR expression and circulating suPAR levels were initially described as prognostic factors in various forms of malignancies and has later been described as a marker of immune activation, disease severity and prognosis in multiple inflammatory and infectious diseases.1,4-8

Rheumatoid arthritis (RA) is an autoimmune disease characterized by joint inflammation and subsequent damage to bone and cartilage, but also systemic inflammation, fatigue, increased risk of cardiovascular disease and other extra-articular manifestations.7 The measurement of anti–citrullinated protein antibodies (ACPA) is a diagnostic tool and their presence associates with an increased risk of early erosions and extra-articular manifestations.10 Other than presence of ACPA, only a limited number of biomarkers have shown potential to prognosticate disease progression in RA.11

A few previous studies have investigated circulating suPAR in rheumatoid arthritis.12-14 In these studies, circulating levels of suPAR were increased in patients compared to healthy controls12-14 and correlated with the number of swollen joints12,13 also among patients with limited disease activity.13 However, none of the previously published studies addressed early RA, i.e. a phase of particular importance to tailor antirheumatic treatment in order to prevent future joint damage and disability.15 To fill this knowledge gap, we aimed to evaluate the potential of serum suPAR as a biomarker of disease progression in recent-onset RA.

MATERIAL AND METHODS
Patients and controls. Patients were included in a Swedish prospective observational early RA cohort denoted TIRA-2, which has been described in detail previously.16 In short, patients with < 12 months symptom duration fulfilling either the American College of Rheumatology 1987 (ACR87) criteria (96%) or a clinical RA diagnosis based on symmetrical small joint arthritis and >60min morning stiffness (4%) were enrolled and followed prospectively.16 Serum samples and clinical variables were collected at inclusion and at 3, 6, 12, 24 and 36 months of follow-up.

### Table I. Baseline characteristics of the 252 early rheumatoid arthritis patients

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Mean (range) or number (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>58 (19-85)</td>
</tr>
<tr>
<td>Female</td>
<td>179 (71)</td>
</tr>
<tr>
<td>RF positive</td>
<td>141 (56)</td>
</tr>
<tr>
<td>ACPA positive</td>
<td>173 (69)</td>
</tr>
<tr>
<td>Smoking</td>
<td></td>
</tr>
<tr>
<td>Never</td>
<td>42 (28)</td>
</tr>
<tr>
<td>Current</td>
<td>38 (26)</td>
</tr>
<tr>
<td>Previous</td>
<td>59 (40)</td>
</tr>
<tr>
<td>Irregular</td>
<td>8 (5.4)</td>
</tr>
<tr>
<td>Non-cigarette smokers</td>
<td>2 (1.2)</td>
</tr>
<tr>
<td>Shared epitope carriage (≥ 1 copies)</td>
<td>187 (75)</td>
</tr>
<tr>
<td>Larsen score</td>
<td>2.5 (0-23)</td>
</tr>
<tr>
<td>DAS28</td>
<td>5.1 (2.0-8.1)</td>
</tr>
<tr>
<td>Methotrexate initiated</td>
<td>227 (90)</td>
</tr>
<tr>
<td>Oral corticosteroids initiated</td>
<td>159 (62)</td>
</tr>
</tbody>
</table>

Abbreviations: ACPA, anti-citrullinated protein antibodies; DAS28, 28-disease activity score; RF, rheumatoid factor.

1 Available for 238 patients.
2 Available for 149 patients. The patients were classified as current smokers if smoking cigarettes regularly within one year of inclusion.
3 Available for 250 patients.
4 Available for 231 patients.
5 Available for 236 patients.
Larsen score was recorded at inclusion and at the 36 months follow-up visit. In this study, 252 patients were analyzed for serum suPAR at three time points (0, 3 and 36 months). Inclusion samples and 3 months samples were chosen to represent the dynamic first phase of the disease where changes in disease activity are most likely to appear. Long term effects were studied at 36 months.

No patient had received disease-modifying anti-rheumatic drugs prior to the initial blood sampling at the first visit (baseline). Baseline characteristics are shown in Table I.

ACPA was analyzed by fluorescence enzyme immunoassay on the Phadia 250 instrument with 2nd generation cyclic citrullinated peptides (CCP) as antigen (EliA; Thermo Fisher AB, Uppsala, Sweden). Rheumatoid factor (RF) assessments were performed locally, by agglutination test, according to clinical routine at the respective participating rheumatology unit. Details about shared epitope genetics are described elsewhere.

One hundred healthy blood donors (49 men, 51 women; mean age 43.6 years; range, 21-71 years) served as control subjects.

Peripheral venous blood was drawn from each individual at every visit. Serum was prepared and stored at -70 °C until analysis.

Ethics. This study was carried out in accordance with the Declaration of Helsinki. Written informed consent was obtained from all subjects. The TIRA-2 study was approved by the regional ethical review board in Linköping, Sweden (Decision No. M168-05/2006).

Clinical variables. The patients were prospectively followed with regular assessments of 28-joint Disease Activity Score (DAS28),18 erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), radiographic joint damage according to the Larsen method,19 and information regarding ongoing medication. A 3-year radiographic progression score was calculated by subtracting the Larsen score at baseline from the Larsen score at the 36 months follow-up visit.

suPAR measurements. suPAR was measured using the human uPAR duoset enzyme-linked immunosorbent assay (ELISA) (R&D systems, Abingdon, United Kingdom). This ELISA contains a monoclonal capture antibody generated against an amino acid sequence found on each of the suPAR subunits (D1, D2 and D3) which is combined with a polyclonal detection antibody. Considering the potential interference of autoantibodies directed towards the Fc-part of IgG (i.e. rheumatoid factors; RF) with suPAR detection in the ELISA, suPAR spiking was performed in two RF positive and two RF negative samples. Recombinant suPAR (standard from the uPAR duoset ELISA) was added in 2 different concentrations to the respective serum sample, and the sera was thereafter analyzed by the ELISA assay. The recovery was comparable between RF+ (range 80-113%; mean 98%) and RF- sera (range 85-93%; mean 88%) (Supplementary Table 1).

Sera from patients and controls were diluted 1:10, 1:15 or 1:20 and analyzed as duplicates according to manufacturer guidelines (R&D systems). For every patient, samples taken at 0 and 3 months were assayed on the same plate to avoid interassay variation. Samples from the 36 months visit were analyzed separately from months 0 and 3. However, values from all samples were normalized according to an internal control that was assayed on every plate. If coefficient of variation (CV) values of assay duplicates differed more than 20%, samples were rerun. Details and raw data from the ELISA are found in Supplementary data 1. Absorbance was measured at 450 nm using Sunrise microplate reader (Tecan Austria GmbH, Grödig, Austria) and data were processed in Magellan software version 7.1 (Tecan).

Statistics. Age, suPAR and DAS28 had a Gaussian distribution at all three timepoints whereas CRP, ESR, Larsen score, number of swollen joints, number of tender joints and 3-year radiographic progression were generally not normally distributed. Therefore, students-t test or One-way ANOVA was used to compare suPAR levels, age and DAS28 in two and three groups, respectively. The repeated measures ANOVA was performed to compare suPAR levels among the patients at the three different time points. When this ANOVA was statistically significant, the Tukey-Kramer post hoc test was performed, comparing all groups with each other. For comparisons between patients and controls we used ANOVA with Dunnett’s post hoc test. Comparisons regarding CRP, ESR, Larsen score or 3-year radiographic progression were performed with Mann-Whitney U test. All correlations were evaluated using Spearman’s correlation for comparability. Multiple linear regressions were validated by controlling the normality of residuals, linearity of the model, absence of autocorrelation (Durbin-Watson; value between 1.8 and 2.2), collinearity (VIF<2 and Tolerance>0.5) and by detection of outliers (scatter dot of Leverage vs DfFit).

A two-tailed P value lower than 0.05 was considered statistically significant. All statistical analyses were performed using GraphPad Prism version 8 (GraphPad Software, San Diego, CA) or SPSS Statistics 19-20 (IBM, Armonk, NY, USA) software.

RESULTS

suPAR levels and subject characteristics over time. Mean suPAR levels among patients differed significantly between all time-points analyzed (Fig 1A), with
the highest mean suPAR levels found 3 months after inclusion (8.47 ng/mL) and the lowest at 36 months (7.14 ng/mL) (Fig 1A). Mean suPAR levels were significantly higher in RA patients at all three visits ($P < 0.001$) compared to healthy controls (Fig 1A). ESR, CRP and DAS28 decreased significantly between inclusion and three months ($P < 0.001$). Changes of suPAR, DAS28 and Larsen score over time are shown in Fig 1B.

There were no significant differences in suPAR levels between men and women among patients at any timepoint. Among the controls, women had higher suPAR levels (mean 4.20 ng/mL) compared to men (mean 3.02 ng/mL; $P = 0.005$). A significant correlation between age and baseline suPAR was found for the patients ($r = 0.227; P < 0.001$) but not among controls.

Mean baseline levels of suPAR were lower in ACPA positive patients compared with ACPA negative patients (7.44 vs 8.86 ng/mL; $P = 0.001$) and levels of ACPA showed a weak negative correlation with baseline suPAR (rho = -0.167, $P = 0.008$). No statistical significance in suPAR levels was found between RF-positive and RF-negative patients.

No difference in baseline suPAR was found dependent on cigarette smoking. However, current smokers had significantly higher suPAR levels at 3 months (mean = 9.38; compared with the remaining patients where smoking status was available (mean = 8.00 ng/mL; $P = 0.016$) as well as compared with never smokers (mean = 7.93; $P = 0.048$). Levels of suPAR were also increased at 36 months among current smokers (mean = 8.25 ng/mL) compared with the remaining patients (mean = 6.65 ng/mL; $P < 0.001$) or never smokers (mean = 6.60; $P = 0.001$). Patients carrying shared epitope (one or two alleles) did not differ significantly regarding mean suPAR levels at any timepoint compared to patients without shared epitope.

ASSOCIATIONS OF SUPAR AND CLINICAL OUTCOME IN RA PATIENTS

Correlation analyses were performed between serum suPAR levels at all three timepoints versus clinical variables (CRP, ESR, DAS28, Larsen score and radiographic progression over 3 year) at the same timepoints (Supplementary Table 2). Correlations were seen between suPAR and DAS28 at baseline ($P < 0.001$, rho = 0.246), as well as between suPAR and Larsen score at 36 months ($P = 0.001$, rho = 0.240). We also performed correlation analysis between baseline suPAR and baseline DAS28 among the patients with 36-months radiographic data available (n = 176), and this revealed a similar correlation ($P = 0.001$, rho = 0.263) as for the whole group of patients (Supplementary Table 2).

Furthermore, changes in suPAR level between 0 and 3 months, 0 and 36 months as well as 3 and 36 months were correlated with clinical outcome variables, but no significances accompanied with a rho>0.2 were found.
Furthermore, baseline suPAR or 3 months suPAR did not correlate with 36 months Larsen score or radiographic progression. We then divided patients into three groups (as equal sized as possible) according to DAS28 and Larsen score, respectively, at baseline and 36 months (Fig 2). The group with the highest baseline DAS28 had significantly higher suPAR levels at baseline. In addition, patients with Larsen score $>5$ at 36 months had higher levels of suPAR at 36 months compared to patients with Larsen score $<2$. No significant differences in suPAR were seen based on Larsen score at baseline or DAS28 at 36 months.

The associations between suPAR and clinical outcomes are independent of age and ACPA. The association between suPAR and DAS28 at baseline was tested in linear regression models. suPAR levels at baseline differed according to ACPA status, thus an adjusted model with suPAR and ACPA was created revealing that suPAR was associated with DAS28 independent of ACPA status (Table II). Next, variables with potential effect on DAS28 was tested one by one in a linear

<table>
<thead>
<tr>
<th>Dependent variable</th>
<th>Model</th>
<th>Independent variables</th>
<th>Standardized Beta</th>
<th>P value</th>
<th>Model $R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAS28 M0</td>
<td>Only suPAR (n = 236)</td>
<td>suPAR M0</td>
<td>0.21</td>
<td>0.001</td>
<td>0.04</td>
</tr>
<tr>
<td>DAS28 M0</td>
<td>Adjusted (n = 236)</td>
<td>suPAR M0 ACPA positive M0</td>
<td>0.19</td>
<td>0.003</td>
<td>0.05</td>
</tr>
<tr>
<td>DAS28 M0</td>
<td>Optimized (n = 236)</td>
<td>suPAR M0 Age</td>
<td>0.17</td>
<td>0.009</td>
<td>0.082</td>
</tr>
</tbody>
</table>

Abbreviations: ACPA, anti–citrullinated protein antibodies; DAS28, 28-joint disease activity score; M0, 0 months (inclusion); suPAR, soluble urokinase plasminogen activator receptor.

One outlier (high suPAR) was found when validating these models. Removal of the outlier resulted in a lower $p$-value for suPAR in all three models.
regression (age, sex, shared epitope, ACPA and smoking status) with DAS28 as the dependent variable. Except for the association between suPAR and DAS28, only age was significantly associated with DAS28 among the tested variables. Based on this, an optimized model was created with age and suPAR as independent variables revealing a significant impact of both age and suPAR on DAS28 (Table II).

At 36 months, suPAR was associated with Larsen score at 36 months. Larsen score was categorized into a binary variable (Larsen score 0-5 vs >5) prior to logistic regression analysis (Table III). An adjusted model with current cigarette smoking (baseline) and suPAR (36 months) as independent variables did not reach statistical significance, but data on both smoking and Larsen was only available for 111 patients. Next, variables potentially associated with Larsen score at 36 months were added one by one to a logistic regression. Shared epitope, smoking status and sex did not reach statistical significance, whereas age and ACPA positivity were positively associated with Larsen score >5. An optimized model with suPAR (36 months), age and ACPA-positivity (baseline) as independent variables (n = 176) did not remove the statistical significance for suPAR (P = 0.023) although ACPA positivity (P = 0.007) and age (P = 0.012) also had a significant impact on Larsen score at 36 months.

**DISCUSSION**

This is the first study to address suPAR as a prognostic biomarker in recent-onset RA. We found elevated suPAR levels in untreated recent-onset RA in association with disease activity, and further increasing levels during 3 months of early disease. The association with disease activity at baseline is in line with previous reports on established disease, but increasing levels during the initial 3 months is surprising, as disease activity robustly declines during the same time-period. In a study by Koga et al, baseline suPAR levels were related to anti-TNF treatment outcome among patients with established RA. Interestingly, patients classified as non-responders had higher pre-treatment suPAR levels in comparison with good responders, despite similar pre-treatment disease activity. This may indicate a multifactorial background of increased suPAR levels that goes beyond clinical inflammation.

Previous studies on inflammatory diseases other than RA have highlighted an association between suPAR and present and/or subsequent organ damage. As irreversible radiographic joint damage could be regarded as the RA counterpart to organ damage, we wished to determine its relation to suPAR levels. In contrast to disease activity, we found no association between suPAR and radiographic damage at baseline. However, suPAR levels at the 3-year follow-up independently associated with radiographic damage. A smaller previous study on established RA did not find an association between suPAR and radiographic joint damage, although the x-rays in that study were part of the clinical routine care and not taken systematically.

It should be noted that the ELISA used in the present study (suPAR DuoSet) is from another manufacturer (R&D Systems) compared to the ELISA used in many other studies cited herein (suPARnostic from Virogates). Differences may exist concerning the ability to detect specific subunits of suPAR and suPAR/uPA complexes as well as the robustness of suPAR detection in samples with e.g. increased blood lipids and hemolysis. The assay from Virogates is a monoclonal antibody sandwich ELISA, measuring full-length suPAR and cleaved forms, whereas the DuoSet ELISA from R&D systems uses a monoclonal capture antibody, but a polyclonal detection antibody. Indeed, a comparison between a suPAR assay from R&D systems (Quantikine Human uPAR ELISA kit) and the kit from Virogates (suPARnostic) generally showed higher levels of suPAR and better discrimination of disease (focal segmental glomerulonephritis) when the suPARnostic kit was used. However, differences between disease groups and healthy controls were detected by both assays.
The presence of ACPA is a risk factor for radiographic progression in RA which is seen also in the current patient cohort. Therefore, it is somewhat surprising that suPAR levels are lower in ACPA positive patients at baseline, while suPAR at 3-years reflects joint damage. Although there is a tendency in contemporary RA that ACPA negative patients have higher disease activity at the time of diagnosis compared to ACPA positive patients, the association between suPAR and disease activity remained significant also after adjusting for ACPA. It is well known that ACPA positive RA have distinct genetic and lifestyle risk factors compared to ACPA negative RA, and possibly, suPAR is a more prominent feature of the latter.

Limitations of this study includes the missing radiographic follow-up data, causing a reduced patient group available for analyses of suPAR in relation to structural joint damage. Nevertheless, the association between DAS28 and suPAR at baseline remained also when analyzing only patients with radiographs available. There is also a lack of disease controls, precluding comparisons concerning changes in suPAR over time with other rheumatic or inflammatory diseases.

In this study, we disclose a time-dependent relationship between suPAR levels, disease activity and irreversible damage in a chronic inflammatory disease. Initially, suPAR associates with RA disease activity but following treatment and clinical improvement, suPAR instead reflects irreversible structural joint damage. These findings are important for upcoming studies on suPAR also in diseases other than RA, highlighting the need for longitudinal studies. The data does not, however, imply an obvious clinical value of suPAR analyses for prognostic purposes in early RA patients.

In conclusion, suPAR levels are elevated throughout 3 years of early RA while associations with disease activity and structural joint damage differ over time. Thus, timing is important when investigating suPAR as a biomarker in inflammatory diseases.

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SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.trsl.2021.02.007.

REFERENCES