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Analytical, biochemical and clearance considerations of soluble urokinase plasminogen activator receptor (suPAR) in healthy individuals

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ABSTRACT

Background: Soluble urokinase plasminogen activator receptor (suPAR) is an emerging marker of cardiovascular disease burden. Appropriate assessment of assay performance and reference interval are required to enable interpretation of results to facilitate its clinical application.

Methods: suPAR was measured using the suPARnostic® ELISA in 155 healthy volunteers. Assay performance was assessed for anticoagulant effect, recovery, interference, linearity and cross-reactivity. The identity of immunoreactive suPAR was confirmed by size-exclusion HPLC. To establish anatomical sites of release and uptake, we measured suPAR in regional samples from subjects undergoing cardiac catheterization.

Results: The median concentration of suPAR was 2.1 ng/mL (IQR:1.7–2.3) in health. In comparison with EDTA, suPAR measurements were affected by lithium heparin (> 10% change) and increased with serum usage. suPAR reactivity also increased in the presence of haemolysis (10 g/L), but was suppressed with urokinase and lipids (4 g/L). In multiple regression analyses, suPAR associated independently with body weight, NT-proBNP and MR-proADM (P = .03) for healthy individuals. Regional plasma sampling showed lower suPAR concentrations in the coronary sinus and renal vein compared with concentrations in femoral arterial samples. Immunoreactive circulating suPAR species had M_r of 10–39 kDa.

Conclusion: The suPARnostic® assay performs acceptably for a clinical assay but is limited in the presence of high levels of hemolysis, lipids and urokinase. We provide the first evidence for the heart and kidneys as organs of suPAR clearance in humans. Additional investigations are warranted to determine whether there is a need to compare the marker performance of differing circulating forms of suPAR.

1. Background

Recent evidence supports the role of soluble urokinase plasminogen activator receptor (suPAR) as an important marker of cardiovascular disease (CVD) burden. It has been known for some time that increased concentrations of suPAR are predictive of outcomes in patients with cancer [1,2], infectious disease [3,4] and in those at high risk of chronic kidney disease [5]. The extended discovery that suPAR also has excellent potential in prognosticating high mortality rates in CVD [6] and in CVD worsening [7,8], is underpin by its ability in retaining similar extracellular function to its cell bound pleiotropic receptor uPAR (NCBI:AAK31795) which is present in a variety of cell-types [9]. The actions of uPAR, highly expressed during immune system dysfunction, include; activating plasminogen conversion to the proteolytically active plasmin through localization of urokinase, and as an orchestrator in dynamic substratum interactions such as cell adhesion, extracellular matrix degradation and in cytoskeletal reorganization [10,11]. These controlled actions are instrumental in promoting atherogenesis and in causing plaque vulnerability [12], and thus the measurement of suPAR is highly suggestive to correlate greater with CVD related burden than other inflammatory markers which can be confounded by obesity [13].

With growing interest in suPAR to potentially add to guiding personalized cardiovascular risk assessment and treatment, it is important to clarify analytical, biochemical, and performance characteristics of available assays, and to understand sources contributing to suPAR bioavailability. Several methods of suPAR measurement are available [14–16], however, one well used assay in clinical populations and the only CE-IVD certified commercially kit available is the suPARnostic®

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two-site enzyme-linked immunosorbent assay (ELISA) from ViroGates, (Birkerød, Denmark) [17]. Apart from reported stability and the effect of sample handling on suPAR measurements [14,18], there is limited information on other key characteristics of this assay. Another important aspect of assay characteristics worth consideration too is in understanding the measuring capabilities of assay formats. In this context, suPAR can circulate either as a full length protein (DI–DIII) or in the truncated forms of DI and DII–DIII. Given that the suPARnostic® assay detects DIII of suPAR [14], implies that it should be capable of recognizing both the full length and DII–DIII but not in distinguishing the amount of the different forms in circulation.

Accordingly, we have used the suPARnostic® ELISA to (i) validate collection and analytical parameters relevant to suPAR measurement, ii) document a suPAR reference interval using samples from 155 well-defined healthy subjects, (iii) characterize the molecular forms of immunoreactive suPAR in circulation as recognized by the assay and (iv) document trans-organ arterial and venous concentrations in catheterized patients to identify anatomical sites of production and clearance of suPAR. Our findings are reported here.

2. Materials and methods

2.1. Human plasma sample collection

Plasma samples were obtained from; (i) healthy volunteers aged 17 to 70 years with no evidence of cardiovascular, endocrine or psychiatric illness and taking no medications (n = 155), (ii) in 7 additional healthy volunteers where within-individual suPAR variability were assessed, (iii) patients admitted to hospital with suspected acute heart failure (HF) (n = 4), and (iv) patients undergoing clinically indicated cardiac catheterization (n = 15). All patients had blood drawn into chilled vacutainer tubes without gel, centrifuged and stored at −80 °C (unless specified) prior to analysis, apart from group (ii) in which suPAR measurements were also performed in a set of fresh plasma. For group (i), a brief physical examination including anthropometric measurements – weight, height, and blood pressure (BP) readings were undertaken by trained staff. Samples were collected by standard venepuncture between 7.30 and 10 am from fasting individuals. For group (ii), blood was drawn daily at 9.30 am for 5 days in 5 healthy individuals. In those same individuals, blood was additionally collected at 9.30 am, 10.30 am, 12 pm, 1 pm and 2.30 pm at one particular day. In 2 additional subjects, bloods were also taken at midnight, 2 am, 4 am, 6 am, 8 am, 10 am and lastly at 12 pm. For group (iii), patients had blood samples drawn immediately at hospital presentation [19]. Patients in group (iv) were catheterized via the left femoral artery and had blood drawn from regional vascular sites as previously detailed [20]. Femoral arterial samples were taken at both commencement and conclusion of the sampling series to provide arterial concentrations and to check for any short term drift in analyte levels. All patients gave informed signed consent to participate in the studies which were approved by the New Zealand Health and Disability Ethics Committee.

2.2. Laboratory measurements

Determination of suPAR reference interval (normal range) was conducted according to CLSI/IFCC guidelines using a direct sampling approach. Standard laboratory measurements (electrolytes, creatinine, lipid profiles, glucose) were performed on fresh lithium heparin plasma and whole blood EDTA [glycated haemoglobin A_{1c} (HbA_{1c})]. Estimated glomerular filtration rate eGFR was calculated using the CKD-EPI formulae [21] based on creatinine concentrations. N-terminal pro B-type natriuretic peptide (NT-proBNP), high sensitivity cardiac Troponin T (hs-cTnT), mid regional pro-adrenomedullin (MR-proADM) and suPAR were assayed in stored EDTA plasma samples that had undergone < 5 freeze/thaw cycles (methods/analytical data are listed in Supplemental Data). In group (ii), a set of plasma samples were analysed within an hour which was used for comparing fresh suPAR concentrations to that of samples that had undergone multiple freeze-thaw cycles. All samples for suPAR measurements were analysed in duplicate by one operator (JCH) using the suPARnostic® ELISA kit, Lot #US1170 [17]. Any suPAR duplicate results which varied by > 10% were repeated. According to the manufacturer, this assay has a coefficient of variation (CV) ≤ 6% at 2.3 ng/mL, detection limit of 0.1 ng/mL and an assay working range up to 20.8 ng/mL.

2.3. Assessment of assay performance, sample collection metrics and within-individual suPAR variability

We performed inter- and intra- batch CV analyses using the ViroGates curve control and a plasma pool in 11 assay runs over 11 days. We estimated the limit of quantitation (LOQ) as ≥10% variation in repeated suPAR measurements in a set of samples with concentrations ranging from 0.1 ng/mL to 0.5 ng/mL. Linearity (sample parallelism) was determined by testing ViroGates standard A (20.8 ng/mL), recombinant suPAR [Sino Biological (Life Technologies) Ref: 10925-H08H-50] (20.0 ng/mL) and sample from a HF patient with a high suPAR concentration (19.0 ng/mL); each to the following dilutions; 1:2, 1:4, 1:8, 1:16 and 1:32 in assay buffer. The effect of collection anticoagulants were assessed by comparing suPAR concentrations in fresh EDTA plasma (reference) with serum and lithium heparin (n = 5) as comparators. Similarly, to assess the effect of freeze/thaw on suPAR concentrations, fresh EDTA plasma (n = 5) was compared with plasma samples which had undergone multiple freeze/thaw cycles up to 5 cycles. For the assessment of daily suPAR variability and within-individual day variability, samples were assayed in the same batch.

To minimise matrix effects in the interference studies, the addition of all exogenous materials/interferent was conducted in a ratio of ≤1:10 dilution of plasma, followed by suPAR measurement. Sample recovery was assessed by spiking three patient's EDTA samples with standard A (ViroGates) and separately with recombinant suPAR. We tested the effect of haemolysis (up to 10 g/L), lipaemia [Intralipid solution Frisenius Kabi, Batch: 801G2069 (up to 6.0 g/L)], and icteria [(Bilirubin, Sigma Aldrich, Lot: 88C-0511) (up to 800 μmol/L)] on suPAR sample performance. To assess the effect of urokinase binding to suPAR immunoreactive measurements, we added human urokinase (Abcam, Cat: ab51966) (100 ng) prior to measurement; to (i) 10 ng/mL of recombinant suPAR in assay buffer, (ii) three samples from healthy individuals and (iii) in three samples from patients from the acute HF cohort. For the latter three patients, high-performance liquid chromatography (HPLC) was also performed on their plasma samples without urokinase addition (described in Section 2.4). The impact of interference was calculated by the percentage change of suPAR concentrations with respect to concentrations without interferent. A > 10% change was considered significant. We also tested the specificity of the assay to discriminate between suPAR and other compounds that may cross-react with the assay (Supplemental File).

2.4. Analysis of suPAR forms using HPLC

The molecular weight (M_r) of suPAR was examined using size exclusion HPLC (SE-HPLC). Briefly, raw plasma samples from three patients in the acute HF cohort (as above), recombinant suPAR, and concentrated ViroGates standard A (Supplemental File) were diluted 1:2 in HPLC buffer (20% acetonitrile/0.1% TFA/0.15 M NaCl) and centrifuged prior to analysis. This sample (100 μL) was then injected to a SE-HPLC system with a TSK-GEL G2000SW LC column (7.5 × 600 mm, Toyo Soda, Tokyo, Japan), running in an isocratic gradient of HPLC buffer. The SE-HPLC was calibrated with markers ranging between M_r 12,000–66,000 (Sigma-Aldrich). Fractions were collected at a flow rate of 0.5 mL/min, dried under an airstream at 37 °C after the addition of 10 μL 1% Triton-X 100, followed by reconstitution in 75 μL assay buffer and then re-subjected to ELISA analysis. For

confirmation, we analysed recombinant suPAR using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) alongside M_r markers from Bio-rad (Cat: 161-0375) and separately using Western Blot (Supplemental Data) with M_r markers from Cell Signaling Technology (Cat: 7720). For the Western Blot analysis, we used peroxidase-conjugated mouse anti-human suPAR provided in the suPARnostic® ELISA and precipitation with a tetramethylbenzidine (TMB) substrate for band detection.

2.5. Statistical analyses

Quantitative variables were expressed as medians [interquartile ranges (IQRs)] or means [95% confidence interval (CI)] where appropriate. All statistical analyses were performed using SPSS *v*25 (IBM Corporation) and graphical presentations using GraphPad PRISM *v*7.04 (GraphPad Software, Inc). Normality of distribution was tested using the Shapiro-Wilk test. Student's *t*-test or Mann-Whitney *U* test was used to evaluate differences between two continuous variables as appropriate. Variables that were not normally distributed were log transformed in subsequent analyses. Spearman's rank correlation coefficient was used to investigate associations between suPAR concentrations and other continuous variables. After a selection of "best-fit" variables, multiple regression analyses (backward) were performed to determine variables that were independently associated with suPAR. For the assessment of suPAR individual variation, concentrations at each timepoint were compared using nonparametric Friedman's test and Dunn's post-hoc multiple comparison test. To derive plasma suPAR gradients across tissues, the initial femoral artery concentration was compared with values in femoral, renal and hepatic veins and inferior vena cava. suPAR concentrations in the concluding femoral artery sample were compared with values in the jugular vein, coronary sinus and pulmonary artery. In all analyses, $P < .05$ was considered significant.

3. Results

3.1. Characteristics of the suPAR ELISA

The inter-assay variation of replicate measurements was 3.6% at 3.9 ng/mL (curve control) and 8.0% at 1.8 ng/mL (plasma pool). The intra-assay variation for the curve control and plasma pool was 4.6% and 5.2%, respectively. LOQ concentration was estimated to be between 0.1 and 0.2 ng/mL (near the LOD). Using plasma EDTA as the reference, measured suPAR concentrations were found to increase on average 13.3% when serum was used. For lithium heparin plasma, suPAR measurements decreased (−13.8%) in two healthy volunteers and increased (14.1%) in the other samples. suPAR from human plasma diluted in parallel (Fig. 1A) with the synthetic standard and recombinant standard, confirming the clinically reportable range of suPAR to be linear from 0.2 to 665.6 ng/mL. In comparison to freshly collected EDTA plasma, suPAR concentrations were not found to vary up to 5 freeze-thaw cycles (P = .5) (Fig. 1B).

Recoveries ranged from 82 to 106% with recombinant suPAR and 92 to 102% recoveries with standard A. suPAR concentrations were affected by haemolysis (positive interference at ≥10 g/L) and was suppressed with lipaemia (≥4 g/L). Bilirubin did not significantly interfere with the assay (up to 800 μmol/L). The addition of urokinase to recombinant suPAR sample resulted in nearly complete loss of suPAR immunoreactivity (4% recovery). In 3 healthy individuals and in 3 patients from the acute HF cohort (also subjected to HPLC), suPAR concentrations decreased by an average of 36% after addition of urokinase. No cross-reactivity was found when Creatinine (8.8 mmol/L), Troponin T (14,300 ng/L), NT-proBNP$_{1–76}$ (9320 ng/L), Troponin I (50,000 ng/L), proADM (10,000 ng/L), procalcitonin (10,000 ng/L) and urokinase (10,000 ng/L) were individually assayed. We did not observe suPAR cross-reactivity with most of the medications tested except for very low level cross-reactions with bendroflumethiazide (< 0.0002%), spironolactone (< 0.00005%), metroprolol (< 0.0008%) and candesartan (0.07%).

3.2. Reference interval

suPAR concentrations were measureable in all healthy volunteers (above estimated LOQ), and were skewed to the right. The mean for the whole population was 2.1 ng/mL with a 95% central distribution of 1.3 to 3.6 ng/mL. For females, mean suPAR concentration was 2.2 ng/mL (reference interval: 1.3–3.6) and for males, mean suPAR concentration was 1.9 ng/mL (reference interval: 1.2–3.5). Baseline characteristics for the healthy volunteers are presented in Table 1.

Bivariate analysis showed suPAR to correlate with eGFR (Spearman's rho, r = −0.29, P < .0001), age (r = 0.31, P < .0001), weight (r = −0.17, P = .04), NT-proBNP (r = 0.41, P < .0001) and MR-proADM (r = 0.26, P = .002) (Fig. 2A–D). suPAR concentrations were higher in females (P = .005) (Fig. 3A) and in subjects with eGFR < 90 mL/min/1.73 m^2 (P = .004) (Fig. 3B).

As there was no evidence of nonlinearity between variables of interest and suPAR in this study cohort, all multiple regression analyses utilised linear regressions. In a model where age, gender, weight, height, systolic BP, diastolic BP, pulse pressure, eGFR, hypertension, NT-proBNP and MR-proADM were used in the model for suPAR prediction, an adjusted R^2 of 0.28 was obtained. However only weight (P = .03), NT-proBNP (P < .0001) and MR-proADM (P < .0001) were significant as suPAR determinants in backward multiple regression and they accounted for 25% of suPAR variability. There were no differences in suPAR concentrations found within the 5 individuals (P = .2) (Fig. 4A) assessed during the same day or in the 2 individuals assessed overnight (P = .2) (Fig. 4B). For day to day variation, suPAR concentrations, again, showed no dramatic changes (P = .09) (Fig. 4C) from the concentrations measured from the first day.

3.3. Regional plasma level results

In 15 patients with coronary disease but normal ventricular function, undergoing regional sampling during elective cardiac catheterization, suPAR concentrations were normally distributed. Mean regional plasma concentrations of suPAR are shown in Fig. 5. No significant differences in suPAR concentrations were found in the initial compared with the repeat femoral arterial sample. Notable trans-organ gradients indicated renal clearance of suPAR with renal venous plasma concentrations of suPAR some 4% lower than arterial levels (P = .04). Coronary sinus concentrations of suPAR fell significantly below arterial concentrations (> 5.1%, P = .03) indicating cardiac clearance of suPAR also occurs. There were no significant differences in suPAR levels between initial femoral artery and concentrations in the inferior vena cava.

3.4. Molecular weight of suPAR

The predicted M_r of full-length suPAR without glycosylation is 31.3 kDa. SE-HPLC revealed endogenous suPAR in plasma from two patients to comprise one large peak at ~M_r 23.6 kDa, smaller than the predicted full-length monomer (Fig. 6A). In the third patient, suPAR immunoreactivity was broader in nature, with a main peak at 12 kDa. The ViroGates standard A eluted at approximately 24 kDa (Fig. 6B). The recombinant suPAR eluted with one major peak at M_r of 39 kDa and a smaller peak at an estimated M_r of 66 kDa (Fig. 6C). Recombinant suPAR was however expected to elute as a larger molecule because of the presence of the signal peptide and a histidine tag in its composition. Both SDS-PAGE and Western Blot (Fig. 6D and E) confirmed recombinant suPAR to have a M_r between 37 and 46 kDa, but not the 66 kDa peak.

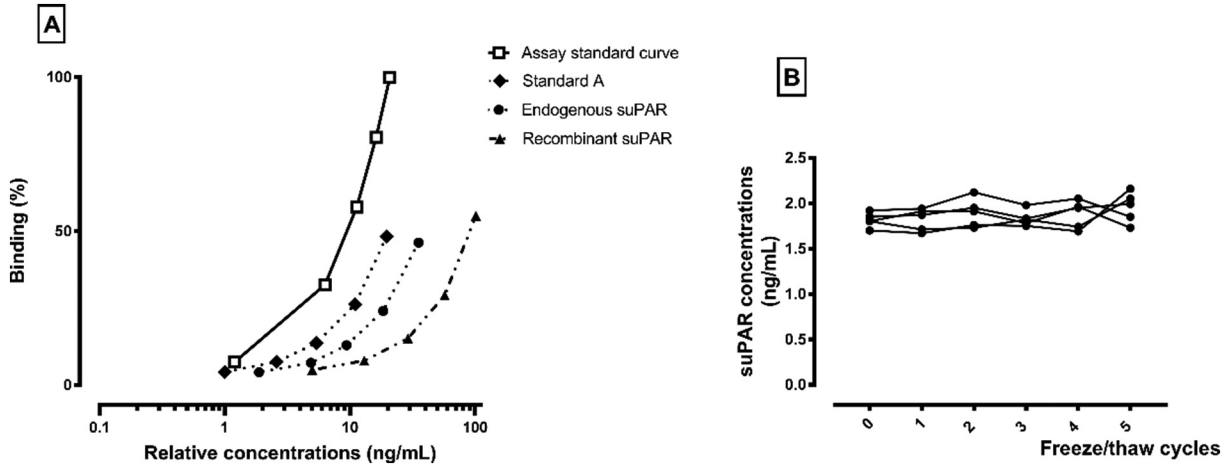

Fig. 1. (A) Total binding for decreasing concentrations of suPAR following dilution with assay buffer for standard A, plasma sample and recombinant suPAR (all dotted lines), compared with assay standard curve (solid line). Regression line for all dilutions were linear on normal scale and the linear regression line for patient plasma, $Y = 0.1897 * X + 0.03612$, $r^2 = 0.9987$. Fig. 1B: suPAR concentrations showing no differences in concentrations between freshly analysed EDTA samples and up to 5 freeze/thaw cycles.

Table 1
Baseline characteristics of enrolled healthy volunteers.

	All volunteers (n = 155)	Females (n = 77)	Males (n = 78)	P value
Age, years	46.8 (31–57)	44.8 (26.5–53.8)	47.0 (34.8–60.0)	0.42
BMI, kg/m^2	25.8 (23.4–28.2)	25.6 (22.1–27.3)	26.9 (25.0–28.8)	0.004
SBP, mm Hg	120 (110–128)	115 (108–126)	120 (111 − 131)	0.19
Weight, kg	79 (66.8–88.9)	68.4 (62.5–77.3)	86.7 (80.8–93.5)	< 0.0001
DBP, mm Hg	80 (70–82)	76 (70–80)	80 (72–88)	0.06
Pulse Pressure, mm Hg	42 (34–50)	40 (34–48)	42 (34–50)	0.98
Hypertension (n)	12	1	11	0.001
Biochemical parameters				
suPAR, ng/mL	2.1 (1.7–2.3)	2.1 (1.8–2.3)	1.9 (1.6–2.2)	0.008
Sodium, mmol/L	142 (141–143)	142 (141–143)	142 (140–143)	0.79
Potassium, mmol/L	4.1 (3.9–4.3)	4.1 (3.9–4.3)	4.0 (3.9–4.3)	0.95
Creatinine, μmol/L	85.0 (76.8–90.0)	78.0 (74.0–85.0)	90.0 (85.0–95.0)	< 0.0001
$eGFR_{CKD\text{-}EPI}$, mL/min/1.73 m^2	84.0 (74.0–94.0)	81.0 (70.0–91.5)	87.0 (75.5–97.0)	0.04
Glucose, mmol/L	5.1 (4.8–5.6)	5.0 (4.7–5.5)	5.3 (5.0–5.6)	0.07
NT-proBNP, pmol/L	4.4 (1.9–8.1)	5.8 (3.3–9.3)	2.4 (1.5–5.6)	< 0.0001
hsTnT, ng/L	6.0 (7.4–7.1) (n = 53)	5.3 (4.1–6.3) (n = 30)	6.6 (4.9–7.4) (n = 23)	0.50
MR-proADM (n = 148),nmol/L	0.44 (0.38–0.52)	0.44 (0.38–0.51) (n = 77)	0.46 (0.38–0.52) (n = 71)	0.19
Cholesterol, mmol/L	5.2 (4.6–5.9)	5.1 (4.6–6.0)	5.3 (4.5–5.9)	0.33
Triglycerides, mmol/L	1.1 (0.8–1.6)	1.0 (0.7–1.2)	1.3 (0.9–1.9)	< 0.0001
HDL, mmol/L	1.35 (1.15–1.56)	1.49 (1.34–1.70)	1.20 (1.08–1.37)	< 0.0001
Calculated LDL, mmol/L	3.3 (2.7–3.8)	3.2 (2.7–3.8)	3.5 (2.7–3.9)	0.47
HbA1c, mmol/mol	33 (30–36)	32 (30–34)	33 (31–37)	0.12

Results are in median (IQR). Abbreviations are as follows; BMI: body mass index, SBP: systolic blood pressure, DBP: diastolic blood pressure, eGFR: estimated glomerular filtration rate, HDL: high density lipoprotein, LDL: low density lipoprotein.

4. Discussion

We report the reference interval of plasma suPAR concentrations in healthy adults. Relationships to demographic, clinical and marker variables are defined and we offer the first examination of multiple trans-organ arteriovenous gradients in plasma concentrations to define sites of production and clearance of suPAR. We also unravel variation in circulating suPAR forms as detected by the ViroGates assay.

When compared to values measured in EDTA plasma, suPAR concentrations were found to have > 10% variation with serum and lithium heparin anticoagulant usage. Increases in suPAR concentrations are to be expected in serum as erythrocyte and leukocyte cell lysis may release suPAR [22]. As lithium heparin inhibits plasmin generation, it was also expected to preserve suPAR concentrations. However our observations showing > 10% changes in both directions could be attributed to other effects of lithium heparin including the destruction of leukocytes, which could also raise suPAR concentrations [23] or alternatively, other matrix interactions which may have resulted in the suppression of suPAR concentrations. This data showing inconclusive effects with lithium heparin use compared with EDTA are thus, at odds with the ViroGates documentation. With respect to stability, we confirmed the findings of other publications which have reported that suPAR is capable of undergoing 5 freeze-thaw cycles in EDTA plasma [14] without variation. Circadian changes in plasma suPAR was also confirmed to be minimal [24,25] in our current study. Although the assay showed expected linearity, recoveries and acceptable CV across its measuring range, it did suffer from positive interference with high levels of haemolysis, negatively with lipaemia, but not by bilirubinaemia (800 μmol/L).

In the assessment of a reference interval, we show suPAR to have a distribution of 1.3 to 3.6 ng/mL in healthy subjects. Bivariate analyses revealed older age, female gender, lower weight, lower eGFR, higher NT-proBNP and higher MR-proADM were associated with higher suPAR concentrations in health. Adjustment by multiple regression showed NT-proBNP, MR-proADM and weight to have independent relationships with suPAR, accounting for 25% of suPAR variability ($P < .0001$). This

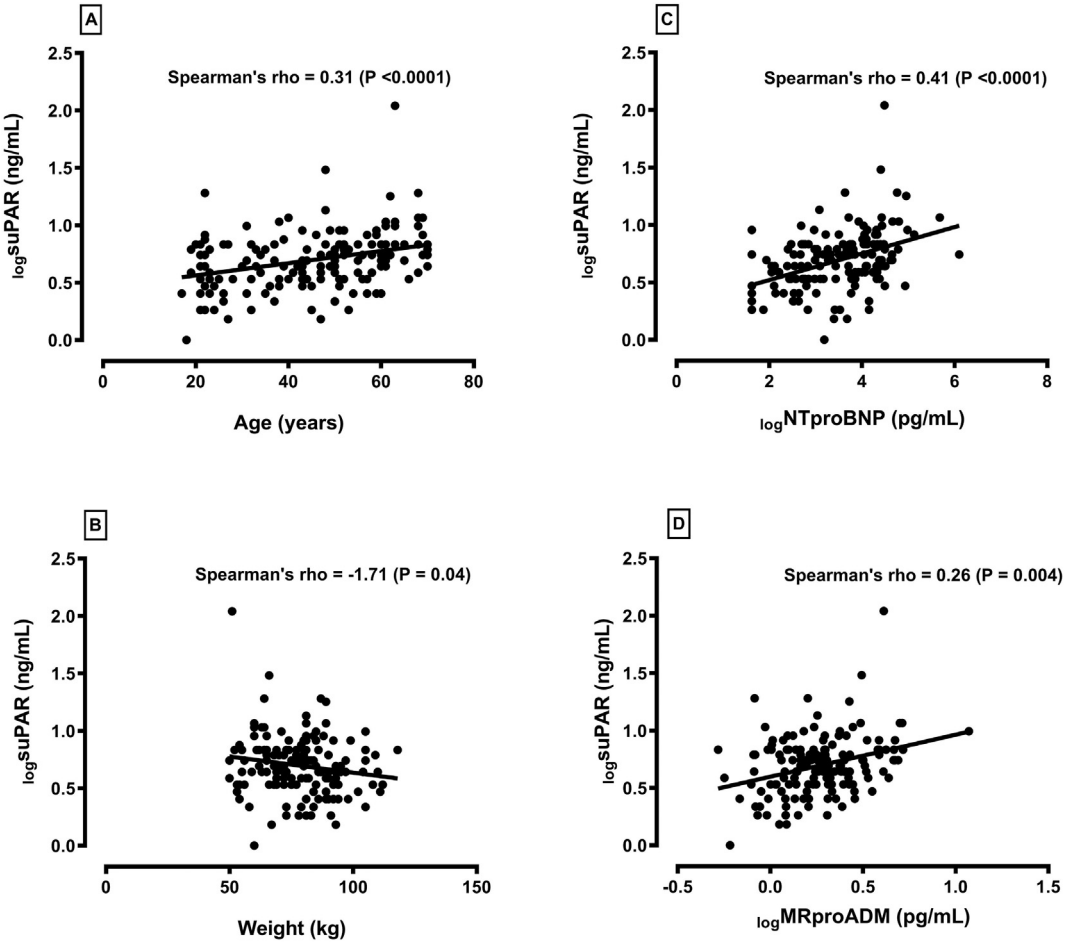

Fig. 2. Correlations of suPAR with age (A), weight (B), NTproBNP (C) and MRproADM (D) in healthy volunteers.

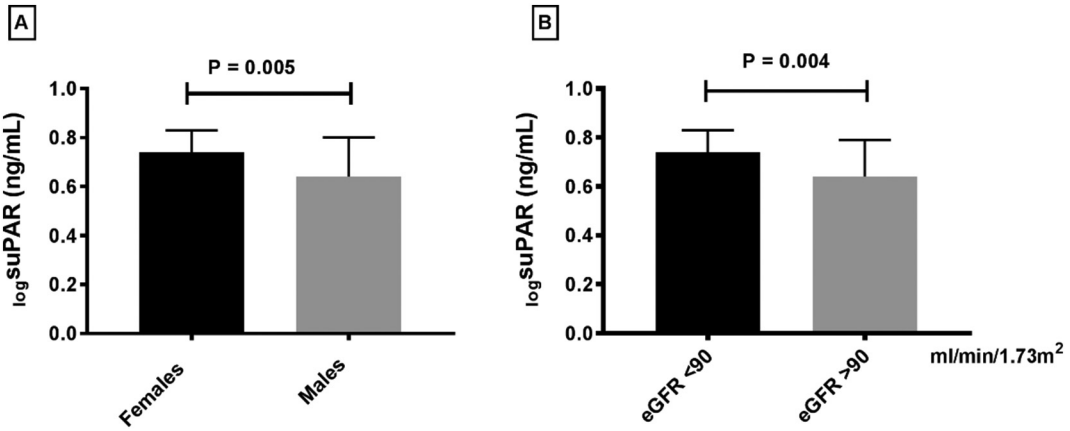

Fig. 3. Median (IQR) log suPAR concentrations for females (n = 77) and males (n = 76) (A), and for individuals with eGFR < 90 mL/min/1.73 m^2 (n = 104) and > 90 mL/min/1.73 m^2 (n = 51) (B). The following suPAR concentrations were obtained for females; median = 2.1 ng/mL (IQR: 1.8–2.3), males; median = 1.9 ng/mL (IQR: 1.6–2.2), individuals with eGFR < 90 mL/min/1.73 m^2; median = 2.1 ng/mL (IQR: 1.8–2.3) and individuals with eGFR > 90 mL/min/1.73 m^2, median = 1.9 ng/mL (IQR: 1.5–2.2).

suggests that there are other determinants of suPAR concentrations yet to be discovered.

To date, several studies reporting on suPAR values in the general population have been published [18,26,27] with median values of suPAR in some studies being slightly higher than ours [27,28]. Our current results are however consistent with previous reports that higher suPAR concentrations are found in females and in older subjects [18,26,28]. Despite this, very little is known on the range and source of variation in circulating suPAR concentrations in health. In females, inflammatory reactions caused by hormonal status may drive higher suPAR concentrations. Similarly subtle increases in systemic inflammation and the incremental loss of kidney function with age may underlie the positive association of suPAR with age. However, in contrast to Haupt et al. [27], our study and others [29] did not find an association between BMI and suPAR. Instead, we found evidence that the inverse correlation of weight with suPAR, remained significant in

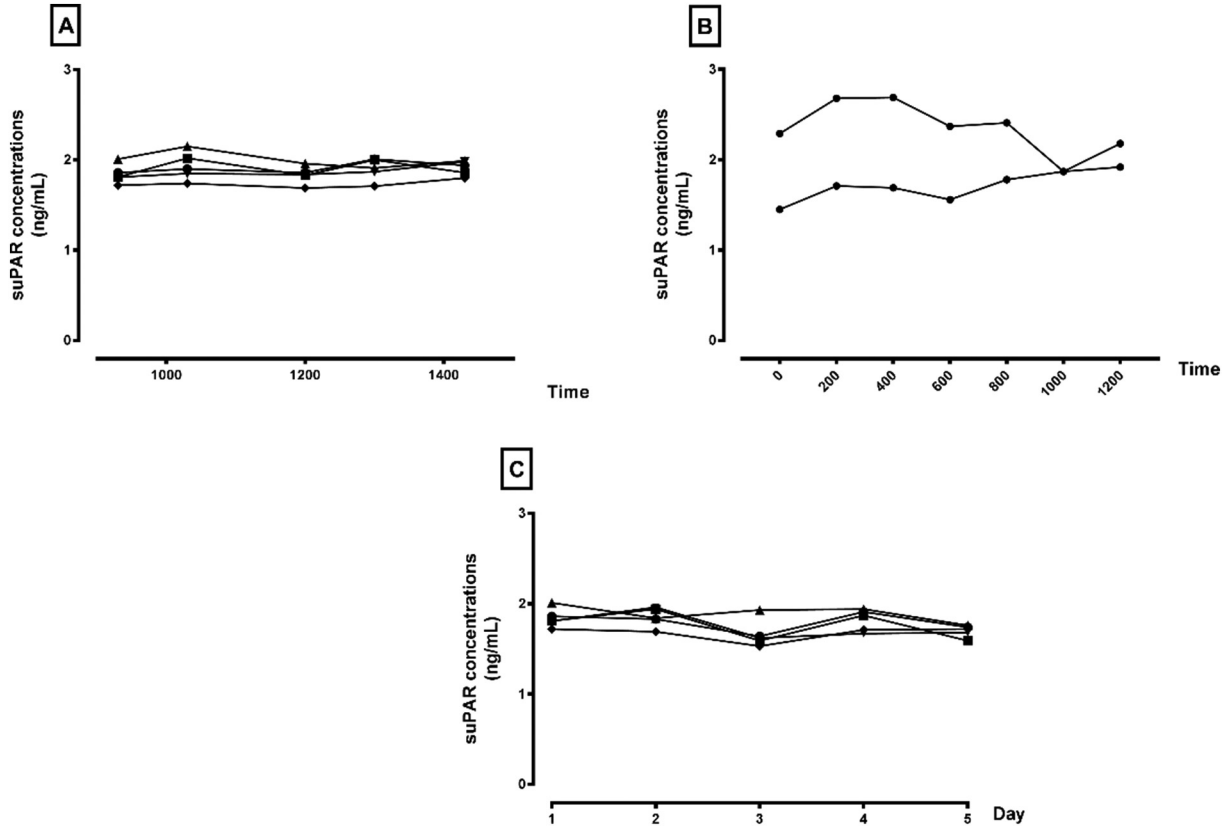

Fig. 4. A; Circadian variation of suPAR in 5 subjects between 9.30 am to 2.30 pm, B; circadian variation of suPAR in 2 subjects between midnight to 12 pm, and C; variation of suPAR in 5 individuals over a period of 5 days.

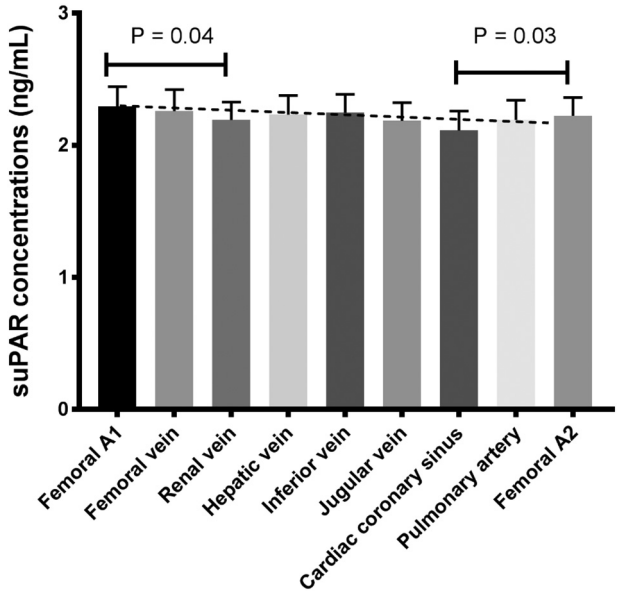

Fig. 5. Regional vascular plasma concentrations of suPAR (ng/mL) [mean (SEM)] in 15 patients undergoing clinically indicated cardiac catheterization.

multiple regression modelling but was not as strongly related as MR-proADM and NT-proBNP. Although speculative, it is conceivable that the inverse relationship of suPAR with weight in the current study may reflect on pro-inflammatory aspects where by internalisation of suPAR (causing lower circulating concentrations) may be upregulated in subjects with higher weight prior to clinical manifestations. Indeed suPAR concentrations, but not CRP, has been found to be highly and independently related to the extent of carotid plaques [13]. However whether lower levels of suPAR in overweight healthy subjects may be attributed to a proatherogenic state (prior to immune system dysfunction when higher suPAR concentrations are raised) requires full consideration and confirmation in future studies involving subjects undergoing longitudinal assessment. Mechanisms underlying the correlation between suPAR and MR-proADM are unclear, but clearly parallels the relationship between suPAR and NT-proBNP [30,31]. MR-proADM is a vasodilatory peptide with cardio-protective effects, including modulation of vascular and cardiac remodelling as well as influencing angiogenesis. Raised levels in the circulation are seen during volume overload (like NT-proBNP) as well as in oxidative or shear stress and in inflammation [32]. The apparent strong association of plasma MR-proADM with suPAR may possibly also reflect a low-grade inflammatory state present in the development of subclinical cardiovascular disease.

To date, suPAR clearance has been suggested as renal [11,33]. That the kidneys could be a clearing agent of suPAR is understandable given that it is a source of urokinase production. Renal clearance is corroborated by the present findings. However, the modest gradient of only 4% maybe a result of a relatively long analyte half-life with much recirculation of immunoreactive suPAR measured by the suPARnostic® assay. Our discovery of a significant fall in circulating suPAR concentrations across the heart indicates cardiac clearance of suPAR. Notably the gradients were assessed in samples from patients with coronary disease and it is uncertain whether similar gradients would be observed in health. Importantly, the findings of strong correlations of suPAR with NT-proBNP in our healthy volunteers, and taken together with the regional sampling dataset, suggest important relationships between suPAR and the heart as well as the kidneys. Contrasting reports suggest urokinase protects cardiac myocytes from oxidative stress [34] whilst others have concluded urokinase to contribute to cardiac fibrosis

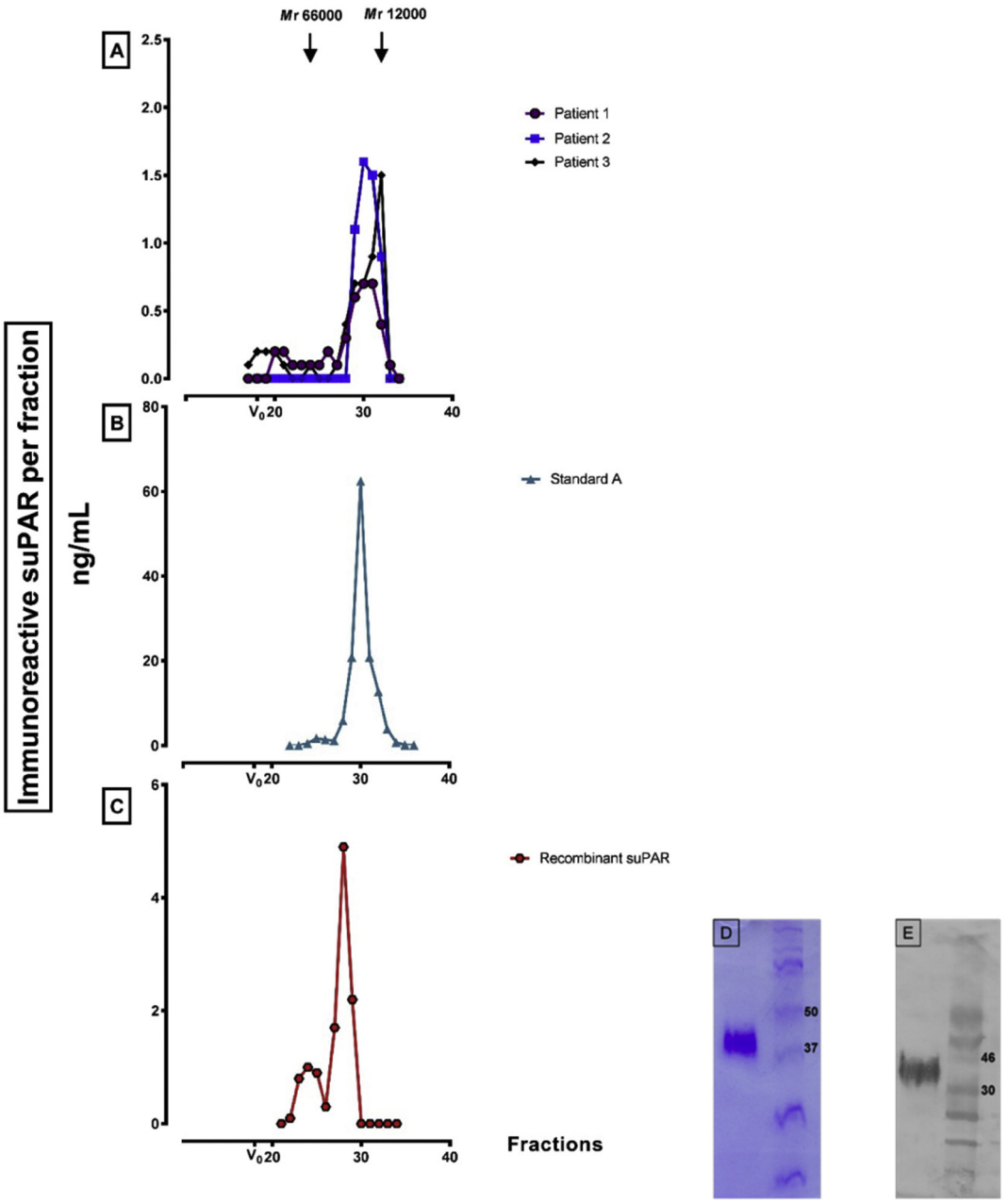

Fig. 6. (A) SEC-HPLC on 3 patients for suPAR molecular weight determinations. Patient 1 is a 74 yr old NZ European male with a BMI of 32.8 kg/m^2, who presented with chronic obstructive pulmonary disorder (COPD), bronchitis, asthma, diabetes, cough and fever, but was not diagnosed with heart failure. suPAR measured with ELISA was 2.8 ng/mL. Patient 2 is a 66 yr old NZ European old male with a BMI of 36.8 kg/m^2, and a history of hypertension, chronic HF and COPD. This patient had a baseline suPAR measurement of 43.3 ng/mL. Patient 3 is a 62 NZ European male who presented to the emergency department with myocardial infarction and a suPAR concentration of 393 ng/mL measured at baseline. Patient 3 also had interstitial lung disease, rheumatoid arthritis and was readmitted to hospital within 90 days. After treatment with polyethylene glycol (PEG), there was < 10% recovery of suPAR for patient 3, whereas in 3 other samples from different patients achieved 104–122% recovery of suPAR after treatment of PEG. This suggests possible interference with suPAR measurement from IgG or Rheumatoid factor (RF). (B) SEC-HPLC for suPAR standard A. (C) SEC-HPLC for recombinant suPAR. (D) SDS-PAGE and (E) Western Blot for recombinant suPAR.

[35]. More work too in this regard is required to determine whether suPAR's prediction of mortality in CVD [8] reflects mechanistic contributions to, or defence from, cardiac injury.

The precise epitope target of DIII from the ViroGates ELISA kit is not disclosed [14,36], but this antibody configuration has been reported to carry the strongest prognostic clinical value [36]. SE-HPLC in the plasma from 2 patients revealed the major circulating form of endogenous suPAR to have an M_r of approximately 23 kDa, and in 1 patient, suPAR circulated as a 10–37 kDa protein, contradicting the predicted 55–60 kDa heavily glycosylated suPAR protein [33,36]. The possibilities explaining this finding include; (1) suPAR from the patients and standard A consisted only of DII-DIII with glycan chains, or (2) degradation of suPAR occurred during HPLC, generating unrecognisable fragments to the assay system but at the same time preserving some DII-DIII fragments that remained immunoreactive. Although a loss of suPAR is to be expected after SE-HPLC, SDS-PAGE and Western Blot performed on the recombinant suPAR (~39 kDa) confirms this full-length M_r that after HPLC, retained its immunoreactivity with the ELISA assay. Full-length suPAR and uPAR with the ligand containing DI region are required for urokinase binding [37]. Given the fact that urokinase addition to patient samples resulted in a > 30% decrease in suPAR signal, implies that full-length suPAR was indeed present in

circulation of patients prior to HPLC, and corroborates the results of others who have found the presence of full-length suPAR in healthy individuals and in disease states [37–39]. The ~30% loss of suPAR after the addition of urokinase to plasma samples further indicates that the majority of suPAR is circulating as truncated forms. Also relevant is the varied epitope recognition by different antibodies targeting specific segments such as that detecting the COOH− of the C-terminal of suPAR DIII [37,40] which is capable of recognizing suPAR bounded to urokinase giving an M_r of > 49 kDa [40]. In the present study, the addition of urokinase to suPAR in plasma must have resulted in a conformational change of suPAR and blocking epitope recognition by the ViroGates antibodies. Given this, we could surmise that the ViroGates assay is only capable of measuring the free form of suPAR. Whether there is a need to detect the different forms of suPAR in circulation in CVD and other conditions also requires further elucidation [41]. Of note, we have also discovered the possibility of interference in suPAR measurements in one of our patients which may be caused by the presence of high levels of IgG or rheumatoid factor concentrations (Fig. 6). The impact of endogenous interfering substances in causing suPAR false-positives also requires further assessments.

5. Conclusion

In conclusion, our study has shown links between suPAR and the heart. suPAR is cleared by both cardiac and renal mechanisms. suPAR is independently related to plasma NT-proBNP and MR-proADM, well recognized markers of cardiac load and clinical risk. Interrogation of samples from cardiovascular disease cohorts will enable further definition of suPAR's performance and potential utility as a prognostic marker in CVD. Our assessment indicates the suPARnostic® ViroGates ELISA performs acceptably for clinical applications. Results are however susceptible to error in the presence of high levels of urokinase which lowers suPAR. Further clarifications are also warranted as to whether lithium heparin and EDTA plasma usage are interchangeable without leading to clinically significant changes in suPAR measurements. Our unique results have shown the presence of circulating truncated forms of suPAR which have not been studied in CVD patients using the ViroGates assay. Our study has advanced the understanding of suPAR biology in identifying additional clearance by the heart in a pathologic population.

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