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Establishing reference intervals for soluble urokinase plasminogen activator receptor in Northern European adults

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ABSTRACT

Objectives: Soluble urokinase plasminogen activator receptor (suPAR) may have untapped potential in clinical diagnostics. Previous studies determined reference intervals using an enzyme-linked immunoassay, but there is a need for reference intervals using a faster assay if the analysis is to be used in emergency medicine. The current study aims to determine reference intervals for suPAR using a fully automated particle-enhanced turbidimetric immunoassay (PETIA) according to the Clinical and Laboratory Standards Institute guideline A28-A3c.

Design and methods: Blood samples were prospectively collected from Danish blood donors. Plasma suPAR was analyzed on the cobas 8000 module c502 in an open channel using a PETIA. Sex-partitioned reference intervals were determined using a parametric quantile approach.

Results: The study included 241 participants—123 females and 118 males. The common reference interval for suPAR was 1.56–4.11 ng/mL (95% confidence intervals (CI) for the lower and upper limits were 1.56–1.63 and 3.81–4.47, respectively). The reference interval for females was 1.59–4.65 ng/mL (95% CIs 1.48–1.70 and 4.09–5.48, respectively) and for males, 1.56–3.59 ng/mL (95% CIs 1.47–1.65 and 3.31–3.93, respectively).

Conclusions: Our results support using sex-partitioned reference intervals for suPAR and provide a basis for future studies using the PETIA method.

1. Introduction

Soluble urokinase plasminogen activator receptor (suPAR) is a protein present in blood and other body fluids. It is the soluble form of the membrane-bound urokinase plasminogen activator receptor (uPAR) [1]. uPAR is expressed in various cells, such as neutrophils, monocytes, macrophages, endothelial cells, fibroblasts, smooth muscle cells, and some tumor cells [2]. uPAR expression increases during inflammation, immune responses, and injury [3]. Growth factors, inflammatory cytokines, lipopolysaccharide, and cell-cell contact stimulate uPAR shedding and, thereby formation of suPAR [4]. suPAR's biological function remains unclear, but it may play a role in immune-cell chemotaxis, angiogenesis, and neutrophil clearance [5–7].

suPAR has recently been subject to increasing interest as a potential biomarker of sepsis in emergency patients [8]. Sepsis is an incompletely understood clinical syndrome characterized by a dysregulated response to infection [9]. In sepsis, serum levels of membrane-bound glycoproteins such as suPAR increase [10]. In an emergency setting, traditional triage combined with suPAR measurement may improve the identification of at-risk emergency patients [11]. Furthermore, suPAR may be associated with reduced

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Abbreviations

CI	confidence interval
CLSI	Clinical and Laboratory Standards Institute
ELISA	enzyme-linked immunosorbent assay
IQR	interquartile range
PETIA	particle enhanced turbidimetric immunoassay
SD	standard deviation
suPAR	soluble urokinase plasminogen activator receptor
uPAR	urokinase plasminogen activator receptor

length of hospital stay [12]. Measuring suPAR levels may have diagnostic value in sepsis and predict sepsis mortality [13,14]. In addition, suPAR is associated with various diseases and has been reviewed as a marker of kidney disease, sepsis, cardiovascular disease, and inflammatory disorders [4,15–18]. Thus, suPAR has an impending untapped potential in clinical diagnostics.

The clinical usage of suPAR may include sepsis detection and monitoring [8]. However, diagnostic usage and method comparison are limited by the lack of relevant reference intervals representing the normal range in healthy individuals. Reference intervals, analytical performance, and biological variation are essential for interpreting laboratory results in everyday clinical practice. Therefore, clinical laboratories and manufacturers must provide accurate and reliable reference intervals. Determination of the reference interval for suPAR may improve the biomarker's usefulness and feasibility in further studies and clinical practice.

Several laboratory methods are available for quantitative suPAR measurement, including an enzyme-linked immunosorbent assay (ELISA) and a particle-enhanced turbidimetric immunoassay (PETIA). The suPARnostic (ViroGates, Denmark) ELISA and PETIA methods are comparable, but the PETIA is much faster than the ELISA, making it more suitable for routine clinical analysis [19]. Previous studies have determined suPAR reference intervals using the ELISA [20,21]; however, the current study is the first to determine reference intervals using the fully-automated PETIA according to the Clinical and Laboratory Standards Institute (CLSI) guideline A28-A3c [22].

2. Methods

2.1. Study design and participants

Participants were consecutively recruited among blood donors at the Danish Blood Services at Biochemistry and Immunology, Lillebaelt Hospital, University Hospital of Southern Denmark, Denmark from 1 April to June 30, 2020. The inclusion criteria for blood donors were age between 17 and 70 years. Exclusion criteria included infectious diseases, severe allergy or asthma, severe chronic obstructive pulmonary disease, autoimmune disorders, cancer, recent surgery, pregnancy, cardiovascular diseases, severe kidney, gastrointestinal or endocrine disorders, anemia, blood disorders, blood transfusion, sexual risk behavior, drug abuse, and weight <50 kg. Additionally, according to the Standards for Transfusion Medicine, donors were excluded if they used any medication incompatible with blood donation [23]. Two hundred forty-three participants were eligible for further analysis.

2.2. Test methods

Blood samples were drawn from the antecubital vein into BD Vacutainer lithium heparin tubes (Becton Dickinson, Franklin Lakes, New Jersey, USA). Next, the samples were centrifuged at 2654 g for 5 min and transferred to new tubes before freezing at $-80\ ^{\circ}C$ until analysis. Plasma suPAR was analyzed on the cobas 8000 module c502 (F. Hoffmann-La Roche Ltd.) in an open channel. The particle-enhanced turbidimetric immunoassay suPARnostic TurbiLatex (ViroGates, Denmark) was used per the manufacturer's protocol. However, the correction factor suggested by the manufacturer when using lithium-heparin tubes instead of EDTA tubes was omitted. According to the manufacturer, the most significant coefficient of variation was 10.3% at a mean level of 3.4 ng/mL. The lower limit of quantification was 1.2 ng/mL, and the method was linear between 1.8 and 26.5 ng/mL [24].

2.3. Statistical analysis

The normal distribution of the suPAR results was assessed using q-q plots and Shapiro-Wilk tests. The results were expressed by the median, interquartile range (IQR), and min-max range. Differences between sexes were estimated with a non-parametric Wilcoxon-Mann-Whitney test. The need for sex partitioning was evaluated according to Lahti et al. [25]. After estimating the common reference interval, the proportions of the two subgroups outside the reference interval were assessed. Subgroup-specific reference intervals were calculated if the proportions exceeded 4.1% or fell below 0.9%. Data normalization was obtained by exponential transformation. Outlier detection was performed using the method proposed by Dixon [26]. A ratio was calculated between D (the absolute difference between extreme observations and the following largest/most minor observation) and R (the range of all observations). If the ratio exceeded 1/3, the observation was considered an outlier. One sample failed during analysis, and one outlier was detected. Both samples were omitted from further analysis. The common, female, and male reference intervals were calculated using a parametric

quantile approach on the exponentially transformed data and back-transformed to the original scale after estimation. Reference intervals were calculated as the 2.5th and 97.5th percentiles with 95% confidence intervals. Statistical analyses were performed using Analyse-it for Microsoft Excel version 2.30 (Analyse-it Software, Ltd. http://analyse-it.com/; 2012). The density plot was produced using R for Windows 4.1.1 (The R Foundation for Statistical Computing, Vienna, Austria).

According to the Danish Act on Research Ethics Review of Health Research Projects, ethical approval was not required as the study was a quality assurance project including anonymized participants.

3. Results

The present study included 241 healthy blood donors. A summary of the suPAR values according to sex is shown in Table 1.

3.1. Distribution

Mean (SD) suPAR values were 2.52 (0.76) combined, 2.67 (0.90) in females, and 2.36 (0.53) in males. The distribution of suPAR values is shown in Fig. 1. Kurtosis and skewness were 18.14 and 3.1, respectively, suggesting a leptokurtic and right-skewed distribution. A significant Shapiro-Wilk test confirmed a non-Gaussian distribution ($p < 0.001$).

3.2. Sex differences

Females and males displayed different suPAR values (Table 1). The proportion of females with results below and above the common reference interval was 2.44% and 3.25%, respectively. The proportion of males with results below and above the common reference interval was 3.39% and 0.85%, respectively. Because less than 0.9% of the male results were above the common reference interval, sex partitioning was warranted.

3.3. Reference intervals+

After exponential transformation, kurtosis and skewness were 0.40 and 0.0, suggesting closeness to a normal distribution, which was confirmed by a Shapiro-Wilk test ($p = 0.155$). The common reference interval for suPAR was 1.56–4.11 ng/mL (95% CIs for the lower and upper limits were 1.56–1.63 and 3.81–4.47, respectively). The reference interval for females was 1.59–4.65 ng/mL (95% CIs 1.48–1.70 and 4.09–5.48, respectively) and for males, 1.56–3.59 ng/mL (95% CIs 1.47–1.65 and 3.31–3.93, respectively).

4. Discussion

In this study, we calculated the suPAR reference intervals in healthy blood donors using a parametric quantile approach on exponentially transformed data according to the CLSI guideline EP28-A3c [22]. We found that sex partitioning was necessary and determined reference intervals for females and males separately.

The medians in the present study are similar to those reported by the manufacturer (2.6 ng/mL in females and 2.2 ng/mL in males). The manufacturer only reported 25–75% intervals in healthy individuals, which are not comparable to actual reference intervals [24]. Two previous studies determined suPAR reference intervals using the suPARnostic ELISA (ViroGates, Denmark) [20,21]. A comparison of the PETIA and ELISA methods showed very similar results with a relative difference of <15%, and the observed correlations between methods were strong ($r > 0.95$) [19]. Chew-Harris et al. investigated suPAR reference intervals in 155 healthy volunteers aged 17–70. The reference intervals were 1.3–3.6 ng/mL in females and 1.2–3.5 ng/mL in males [20]. Like the present study, they found a significant difference between median suPAR levels in females and males. Wlazel et al. determined a suPAR reference interval to be 2.33–6.79 ng/mL in 326 Caucasian elderly (74–88 years old) according to the CLSI A28-A3c guideline [21]. The lower and upper reference limits were significantly higher than in the present study, as confidence intervals do not overlap. The difference may be explained by the fact that the study populations consist of different age groups. They found no difference between sexes in the elderly comparing medians, but there was a significant difference in the younger reference group (24–66 years old) [21]. However, even with smaller differences between medians, previous studies showed that without partitioning, the proportions of a subclass outside the reference interval might be very different from the desired 2.5% on each side [22].

A significant strength of our study is the use of the commercially available suPARnostic Turbilatex reagents, which are validated on the automated cobas 8000 platform and easily implemented in laboratory routines. Moreover, trained laboratory technicians conducted the analyses at an accredited laboratory as batch analyses. The study's primary limitation is the lack of donor data on potential

Table 1
suPAR results for the 241 participants.

	N (%)	Median	Interquartile range	Min-max
Common	241	2.36 ng/mL	2.07–2.81 ng/mL	1.32–8.32 ng/mL
Female	123 (51%)	2.49 ng/mL	2.21–3.04 ng/mL	1.36–8.32 ng/mL
Male	118 (49%)	2.25 ng/mL	2.04–2.61 ng/mL	1.32–4.79 ng/mL

suPAR, soluble urokinase plasminogen activator receptor; IQR, interquartile range.

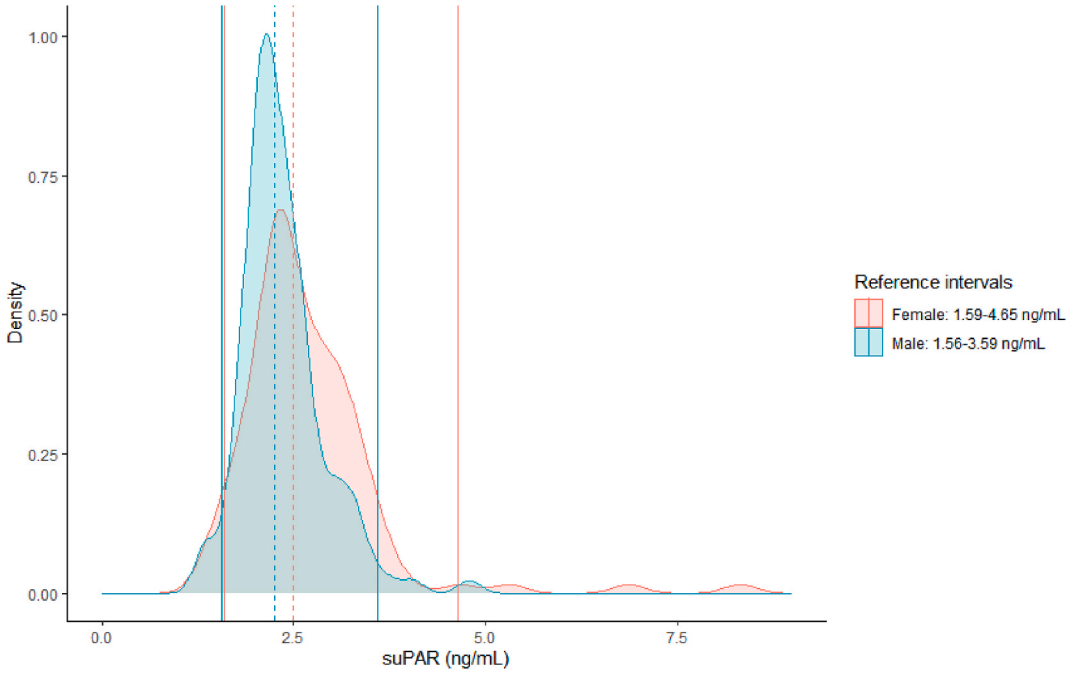

Fig. 1. Distribution of soluble urokinase plasminogen activator receptor (suPAR) values of the 241 healthy blood donors.

confounders such as age, ethnicity, comorbidities, medication, and lifestyle factors that may affect suPAR levels. Indeed, when comparing the elderly to a younger reference group, Wlazel et al. observed higher median suPAR levels in the elderly. C-reactive protein, troponin T, N-terminal pro B-type natriuretic peptide, and estimated glomerular filtration rate also predicted suPAR levels, which may indicate that comorbidities affect suPAR levels [21]. The prevalence of Danish blood donors is relatively constant between the ages of 25 and 55. The potential effect of comorbidities may not be as apparent in Danish blood donors as they are younger than in the study by Wlazel et al. Furthermore, considering ethnicity, minority ethnic groups are underrepresented in Danish blood donors; thus, most participants are expected to be Caucasian [27]. Hence, caution should be taken when assessing suPAR levels in different age groups and ethnic groups.

Blood samples were stored at −80 °C before analysis, which may compromise the analyte's durability. However, a previous study showed that freezing and thawing did not affect suPAR concentrations [20]. Samples were drawn into lithium heparin tubes, and the manufacturer suggests using a correction factor when using lithium heparin tubes instead of EDTA tubes [24]. In the current study, we chose not to use the correction factor because if the analysis were implemented in a laboratory routine, the sample would be obtained using lithium heparin tubes like many of our other acute blood tests. We, therefore, wanted to determine reference intervals for lithium heparin plasma. A previous study showed that suPAR concentrations varied by >10% comparing EDTA and lithium heparin plasma, but there was no clear direction of the variation [20]. Therefore, caution should be taken when comparing studies using different matrices and test tubes for suPAR analyses, even when the correction factor is employed.

Considering implications for practice, the current recommendation from the manufacturer includes three cut-off levels but does not take sex differences into account [24]. Since the present study found a need for sex partitioning, it may be relevant to implement different cut-off values for females and males. The upper reference limit for females is 4.65 ng/mL, and the suggested cut-off for patients of medium risk is 4.0–6.0 ng/mL. Fewer females might be categorized as medium risk if sex-partitioned cut-off values were implemented. The present study lays the foundation for future studies on suPAR. Future determination of reference intervals should include age partitioning. Additionally, establishing suPAR reference intervals for the PETIA is essential for comparing studies employing this method.

In conclusion, we provided reference intervals for suPAR in lithium heparin plasma using the fully automated PETIA according to the CLSI A28-A3c guideline. Our results support using sex-partitioned reference intervals for suPAR, facilitating its use in research and clinical practice.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to

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Data availability

The data presented in this study are available in Supplementary Materials.

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