Inflammation (© 2010)
DOI: 10.1007/s10753-010-9195-2

Urine suPAR Levels Compared with Plasma suPAR Levels as Predictors of Post-consultation Mortality Risk Among Individuals Assumed to be TB-negative: A Prospective Cohort Study

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Abstract—Plasma levels of the inflammatory biomarker soluble urokinase plasminogen activator (suPAR) have been shown to carry prognostic information in various infectious and inflammatory diseases. The present study aimed to compare the prognostic value of urine suPAR (U-suPAR) to that of plasma suPAR (P-suPAR), thereby exploring the possibility of replacing the blood sample with an easy obtainable urine sample. We enrolled 1,007 adults, older than 15 years of age, with a negative TB diagnosis between April 2004 and December 2006. Levels of U-suPAR and P-suPAR were available in 863 individuals. U-suPAR was measured using a commercial ELISA (suPARnostic®). We found that U-suPAR carried significant prognostic information on mortality for HIV-infected subjects with an area under the ROC curve of 0.75. For HIV-negative individuals, little or no prognostic effect was observed. However, in both HIV positives and negatives, the predictive effect of U-suPAR was found to be inferior to that of P-suPAR.

KEY WORDS: aTBneg; U-suPAR; P-suPAR; mortality; prognostic; Guinea-Bissau.

BACKGROUND

Tuberculosis (TB) is an ancient disease, which continues to pose a major public health challenge. Upon a positive diagnosis of active TB, a 6–8-month treatment regime is initiated to cure the patient.

Less focus have been on individuals coming to the health centres presenting respiratory symptoms consistent with TB but who are diagnosed negative for TB, i.e. have negative sputum results on direct microscopy for Acid Fast Bacilli and have normal X-ray. These individuals are sent home without TB treatment. We term these individuals "assumed TB negative" (aTB-neg). We have recently documented that the aTBneg individuals in the current study had a seven times higher mortality rate at 3 months of follow-up compared with the general population and that P-suPAR carries prognostic information on mortality among the aTBneg individuals [1].

Several studies have shown that an elevated plasma level of soluble urokinase plasminogen activator (P-suPAR) is associated with an increased mortality rate in individuals with infectious diseases, including HIV-1 [2, 3], active TB [4] and sepsis [5, 6] as well as in individuals with cardiovascular disease [7] and cancer[8]. P-suPAR levels positively correlate with pro-inflammatory biomarkers such as TNF-α and leukocyte counts [9] and with C-reactive protein levels [10] and are thought to reflect the degree of inflammation and immune activation [3, 11].

SuPAR has been found in urine samples of healthy individuals, cancer patients and HIV-1-infected patients

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Published online: 16 March 2010

[9, 12, 13]. suPAR levels are quite stable in both blood and urine [13, 14] Furthermore, urine suPAR (U-suPAR) levels were strongly correlated with the plasma suPAR (P-suPAR) levels in ovarian cancer patients [15], in patients with acute leukemia [16] and in HIV-1-infected patients [9].

The objective of the present study was to compare the prognostic strength of U-suPAR and P-suPAR and thereby examine whether a urine sample could replace the blood sample in the suPAR test. If so, a simple urine dip-stick could be developed. The specific objectives were first to investigate whether U-suPAR carried prognostic information on mortality risk in aTBneg individuals. Secondly, the correlation between U-suPAR and P-suPAR was evaluated and finally, the prognostic information carried by the two measures were compared.

METHODS

Setting and Patient Recruitment

This prospective cohort study was conducted in an area included in the demographic surveillance system (DSS) of the Bandim Health Project (BHP) in Guinea-Bissau, West Africa. All individuals that live in the study area are registered with an ID-number, and records are maintained of age, gender, ethnic group and socio-economic factors. Censuses are performed at regular intervals and information on pregnancies, births, mortality, and migration is collected. The BHP study area comprises six suburbs that have been followed in epidemiological studies of TB since 1996; thus, this area provides a unique setting for obtaining the longitudinal information needed for a prognostic study.

All patients presenting respiratory symptoms or signs of TB were registered upon admission at the three health centres in the study area or at the National TB Hospital. A study-number was assigned to each patient, and demographic information, including information on residence, was recorded. In addition, blood, urine, and sputum samples were collected. A total of 1,682 adult patients with respiratory symptoms were screened for TB during the study period. Among these, 466 (28%) were diagnosed with active TB according to WHO guidelines [11]. The remaining 1,216 (72%) individuals were diagnosed as TB-negative according to smear and X-ray examination. Among the 1,216 individuals that were aTBneg, identification and follow-up was completed for 1,007 individuals; 98 individuals could not be located due to incomplete or non-existent addresses. An additional 100 individuals were not identified at the house reported by the patient. Seven individuals were known by the people living at the address but the current survival status was unknown. Age was not registered for four individuals. Of the 1,007 individuals with complete follow-up, 863 were included in the present study because they had both a plasma sample and a urine sample (Fig. 1).

Urine and Plasma suPAR Measurements

A minimum of 20 ml of urine were collected into a clean receptacle and transferred to 15 ml tubes for immediate freezing and storage in -20 degrees Celsius freezers at the National Public Health Laboratory. Urine suPAR levels were determined in Hvidovre Hospital, Copenhagen, using the suPARnostic® ELISA kit (ViroGates, Copenhagen, Denmark). The assay comprised plates pre-coated with a catching monoclonal antibody for loading the sample and an HRP-labelled detection monoclonal antibody that was added to the sample dilution buffer. Briefly, 25 µl of urine sample was mixed with 225 µl of dilution buffer and two aliquots (duplicates) of 100 µl each were added to the plates and incubated for one hour. After washing the plates, 50 µl of substrate was added for 20 min and the reaction was stopped with 50 µl 0.5 M H2SO4. Plates were measured at 450 nm in a spectrophotometer. One kit served to process 39 duplicate samples in less than 2 h. The kit intra- and inter-assay variation was 4% and 8%, respectively. The measurement of plasma were described in detail elsewhere [1].

HIV Testing Procedure

Patients provided blood samples at the first point of contact. Patients were informed that their blood sample would be used for suPAR measurements and be stored for future studies. Following approval from the National Research and Ethics committee, HIV testing was carried out retrospectively in all individuals that were aTBneg when serum was available. Blood samples were analysed at the National Public Health Laboratory in Guinea-Bissau. Sera were screened using the Determine™ HIV-1/2 Serum/Plasma Assay (Abbott, List N0. 7D23-13), and reactive sera were confirmed with both the Capillus HIV-1/HIV-2 (Cambridge Diagnostics, Galway, Ireland) and the ImmunoComb® II HIV 1&2 BiSpot Anti HIV 1&2 EIA (Orgenics Ltd, Yavne, Israel). Patients were not contacted following the test and provided with the results

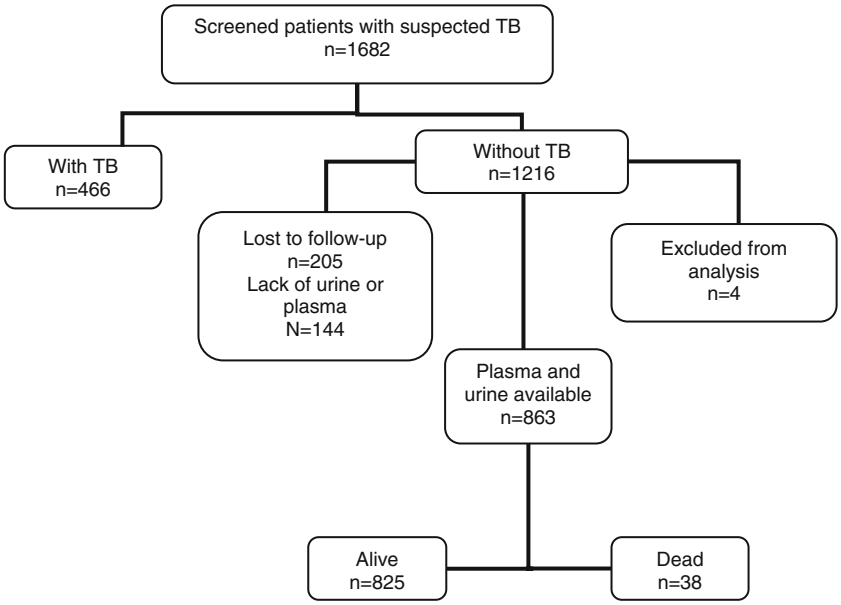

Fig. 1. Flow-chart of included individuals. Loss to follow-up was due to incomplete address, never identified at the address registered, or survival status unknown.

as no anti-retroviral medicine was available during the study period. When anti-retroviral medicine became available in 2007, HIV-positive individuals were invited for a new HIV test and offered ART if their CD4 count was below 350 cells/μl.

TB Diagnosis

TB was diagnosed on the basis of clinical, radiographic, or sputum acid-fast bacilli (AFB) smear results. Three morning sputum samples were collected on 3 consecutive days. The sputum samples were visualized with Ziehl–Nielsen stain and direct microscopy in the health centres or in the National TB Hospital following guidelines from the National Tuberculosis Control Programme. Patients with at least one positive AFB sputum smear sample were diagnosed as smear-positive pulmonary TB (PTB+) cases. If all three sputum samples were smear-negative and the patient continued to have symptoms of TB (fever and cough) after a 2 weeks trial of broad spectrum antibiotics, the patient was referred for X-ray. If clinical signs and X-ray findings were compatible with TB, the patient was diagnosed as smear-negative pulmonary TB (PTB-) case, otherwise the patient was regarded TB negative and included in the aTBneg group; this group constituted our study population. Until 2007, AFB sputum cultures were not routinely performed for individuals suspected to have TB.

Follow-up

During the study period from April 2004 to December 2006, the domiciles of the aTBneg individuals were visited 3 months after study inclusion to ascertain survival. If the individual was not at home, information was obtained from family members or neighbours.

Detailed information on morbidity was not collected. Verbal autopsies were conducted but did not apply to the WHO guidelines.

Statistical Analysis

Data were double entered in a Dbase V database and statistical analyses were conducted in STATA version 10. Time (months) since inclusion was used as underlying time in the Cox-analysis and restricted cubic splines were used to adjust for age. Follow-up was censored at 3 months. The correlation between U-suPAR and P-suPAR was assessed using the correlation coefficient, R^2, and linear regression. The prognostic strength of U-suPAR and P-suPAR was assessed by comparing sensitivity and specificity using c-statistics (ROC curves). The optimal discriminatory suPAR level, giving maximal sensitivity and specificity, was calculated from a Youden index.

Ethical Considerations

All patients provided written consent (or fingerprint) to giving blood and urine and to the use of data for

this study. The Guinea-Bissau Government Ethics Committee and the European Union FP6 Scientific Ethics Committee approved the study protocol, Na. Refa No 011/DHE/2004, and LSSP-CT-2005-012173, respectively. Patients were not initially asked for an HIV test given the fact that there was no HIV treatment available at the time and great stigmatisation of HIV infected. Permission to retrospectively test samples for HIV-1 and HIV-2 was obtained from the Guinea-Bissau National Ethics Committee dated 02, December 2005. When antiretroviral treatment (ART) treatment became available in 2007, HIV-positive aTBneg individuals were re-invited for clinical examination. At this examination, HIV testing was carried out and patients were offered ART based on immunological status (CD4 counts).

RESULTS

Baseline Characteristics

Of the 863 aTBneg individuals included in the analysis, 377 (44%) were males with a median age of 36 years (range, 15–85) and 486 (56%) were females with the median age of 39 years (range 15–79). There was a significant difference between U-suPAR and age ($p=0.004$) and between U-suPAR and HIV-status ($p<0.001$). No significant difference between U-suPAR levels and gender was observed ($p=0.23$). Baseline characteristics according to U-suPAR quartiles are shown in Table 1.

Mortality and U-suPAR

Mortality according to quartiles of U-suPAR is presented in Table 2. Mortality was highest in the 4th U-suPAR quartile (10%). In comparison mortality was 1%, 3% and 4% respectively in the other 3 quartiles. Individuals in the 4th quartile were compared with the remaining individuals in a Cox-model adjusted for age, sex and HIV-status. A mortality rate ratio (MRR) of 3.00 (95% CI: 1.54-5.80) was observed. The ROC curve in Fig. 3, showing sensitivity and specificity for different values of U-suPAR, illustrated a prognostic effect of U-suPAR in HIV-positive individuals while no effect seemed to exist among HIV-negative subjects.

Correlation Between U-suPAR and P-suPAR

Scatter plots of log U-suPAR and log P-suPAR according to HIV-status are illustrated in Fig. 2. Modelling the association between U-suPAR and P-suPAR as linear resulted in a significant positive relationship for both HIV-positive and HIV-negative individuals (both $p<0.001$). The adjusted R^2 was 0.11 for HIV-infected individuals and 0.09 for HIV-negative individuals.

The Prognostic Strength of U-suPAR in Relation to P-suPAR

Table 2 shows the mortality according to quartiles of P-suPAR. Mortality was higher in the 4th P-suPAR quartile (14%) compared with 0%, 1% and 1% in the other 3 quartiles. Individuals in the 4th P-suPAR quartile were compared with the remaining individuals in a Cox-model adjusted for age, sex and HIV-status. A MRR of 11.77 (95% CI: 5.01–27.6) was observed. This was significantly higher than the corresponding MRR of 3.00 for the 4th U-suPAR quartile ($p=0.02$).

The ROC-curves (Fig. 3) illustrated a stronger prognostic effect of P-suPAR compared with U-suPAR

Table 1. Baseline Characteristics of the Individuals that were aTBneg According to Quartiles of Urinary suPAR in ng/ml

Urine-SuPAR Quartiles	1st (0.5–2.9) 215	2nd (2.9–5.8) 216	3rd (5.8–10.5) 216	4th (10.5–53.8) 216	Total (0.5–53.8) 863	*P* value
Age 15–34	111 (13%)	89 (10%)	84 (10%)	98 (11%)	382 (44%)	*P*=0.002
Age 35–54	76 (9%)	74 (9%)	68 (8%)	78 (9%)	296 (34%)	
Age 55+	28 (3%)	53 (6%)	64 (7%)	40 (5%)	185 (21%)	
Male	102 (12%)	100 (12%)	92 (11%)	83 (10%)	377 (44%)	*P*=0.23
Female	113 (13%)	116 (13%)	124 (14%)	133 (15%)	486 (56%)	
HIV-1*	22 (3%)	28 (3%)	46 (5%)	65 (8%)	161 (17%)	*P*<0.001
HIV-2	12 (1%)	20 (2%)	22 (3%)	25 (3%)	79 (9%)	
HIV negatives	178 (21%)	165 (19%)	147 (17%)	122 (14%)	612 (71%)	
No HIV status	3 (0%)	3 (0%)	1 (0%)	4 (0%)	11 (1%)	

Sex and HIV is presented as frequency followed by (%). Prevalence and median age were compared by Chi-square and Kruskal–Wallis tests, respectively

*Includes dual infections of HIV-1 and -2

Table 2. Mortality According to the Quartiles of u-suPAR and p-suPAR

Urine-SuPAR Quartiles	1st (0.1–2.9)	2nd (2.9–5.8)	3rd (5.8–10.5)	4th (10.5–53.8)	Total (0.1–53.8)
Plasma Quartiles					
1st (0.9–2.6)	1/85 (1%)	0/68 (0%)	0/42 (0%)	0/20 (0%)	1/215 (0%)
2nd (2.6–3.3)	0/49 (0%)	2/57 (4%)	0/61 (0%)	1/49 (2%)	3/216 (1%)
3rd (3.3–4.4)	0/49 (0%)	2/53 (4%)	1/58 (2%)	0/56 (0%)	3/216 (1%)
4th (4.4–32.0)	1/32 (3%)	3/38 (8%)	7/55 (13%)	20/91 (22%)	31/216 (14%)
Total	2/215 (1%)	7/216 3(%)	8/216 (4%)	21/216 (10%)	38/863 (4%)

for both HIV-positive and HIV-negative individuals. A test for equal areas under the ROC-curve resulted in respective p values of 0.08 and 0.04 for HIV-positive and HIV-negative individuals.

The optimal discriminatory P-suPAR value was calculated to be 4.4 ng/ml, giving a sensitivity of 0.84 and a specificity of 0.77. The optimal discriminatory U-suPAR level was 7.4 ng/ml, giving a sensitivity of 0.74 and a specificity of 0.61.

DISCUSSION

The aim of the current study was first, to examine if U-suPAR carried prognostic information for mortality, and second, to make a comparison with the information carried by P-suPAR. U-suPAR was observed to carry some prognostic information with a 3 times higher mortality among individuals in the highest U-suPAR quartile. The ROC-curves showed that the prognostic effect of U-suPAR was limited to HIV-infected individuals. Comparing the prognostic strength of the two tests showed a considerably stronger effect of P-suPAR compared to U-suPAR. Nevertheless, U-suPAR did add some value to the plasma suPAR level, as shown in Table 2. Among the 32 individuals who were in the highest P-suPAR quartile, but in the lowest U-suPAR quartile, only one of 32 patients died in the 3-month follow-up period, compared with 20/99 in individuals in the highest U-suPAR and highest P-suPAR quartile.

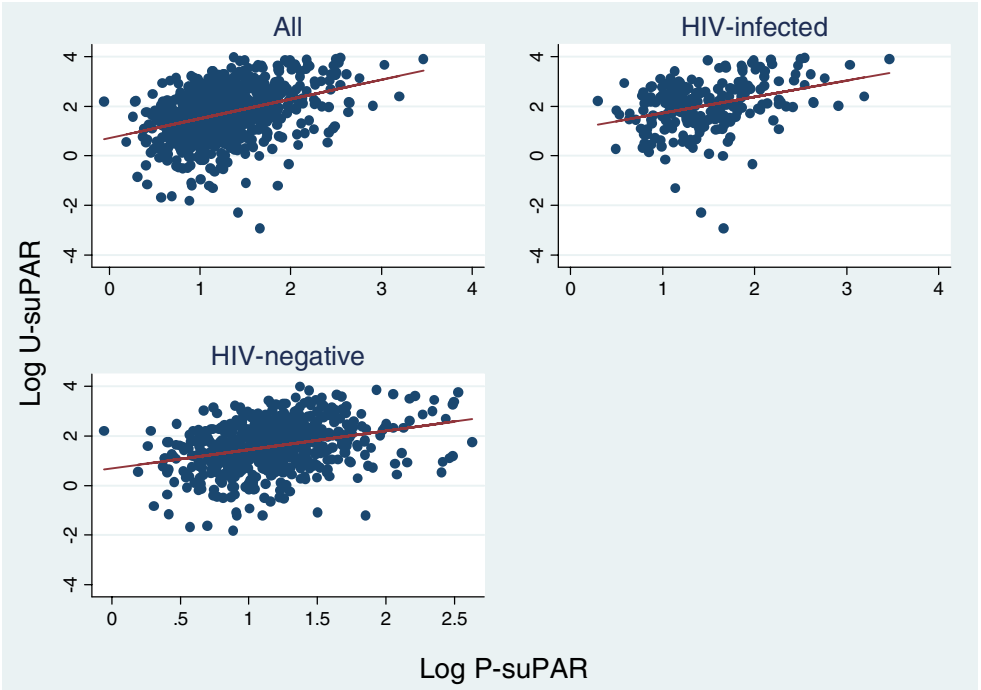

Fig. 2. Scatter plots, according to HIV-status, showing the correlation between U-suPAR and P-suPAR on the log-scale.

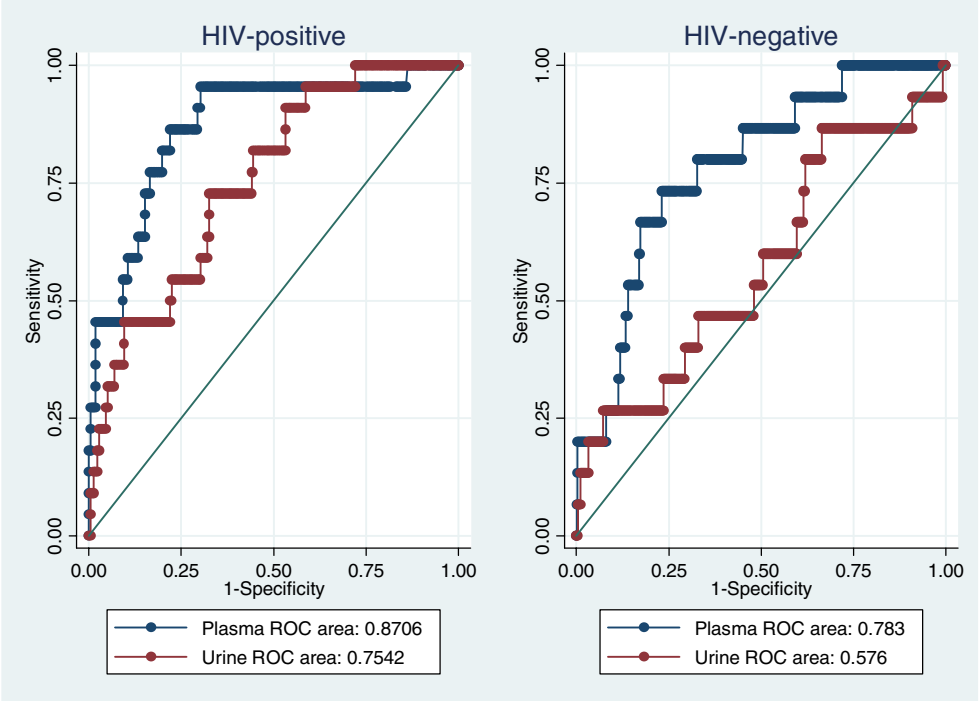

Fig. 3. Receiver operating curves (*ROC* curves) of U-suPAR and P-suPAR.

Creatinine is one of the major components of human urine and it is an end product of muscle metabolism. The creatinine excretion rate into urine is nearly constant, and it is used as an internal standard for normalizing water variation in urine analysis. A previous paper involving 24 HIV-1-positive patients found a stronger correlation between U-suPAR/creatinine ratio and P-suPAR (R^2=0.52)[9]. The major limitation of the present study was the lack of measurements of creatinine content of urine. However, the aim was to investigate whether a simple measurement of suPAR in urine carried prognostic information. If so, a simple lateral flow urine stick with suPAR antibodies could have been developed for post-consultation screening. A lateral flow stick system determining the ratio between U-suPAR and creatinine is more complicated to develop and to apply in clinical practice.

In conclusion our results demonstrated that U-suPAR carries significant prognostic value. However, U-suPAR carried less prognostic value than P-suPAR. Hence, the present study showed that U-suPAR cannot replace P-suPAR as a prognostic test. Whether this will differ with normalized U-suPAR/creatinine ratio can only be examined in further studies.

ACKNOWLEDGEMENTS

The authors are grateful to the staff at the Bandim Health Project, National Public Health Laboratory and Hvidovre Hospital in Copenhagen, Denmark for carrying out patient recruitment, microscopy examinations and suPAR measurements. Further thanks to the staff at Hvidovre Hospital for teaching local Guinean staff the technique of suPAR measurement. We thank the study subjects for their participation.

Competing Interests. Jesper Eugen-Olsen is a shareholder in ViroGates, the company that produces the suPARnostic assay, and has patents on the use of suPAR. The other authors have no conflicts of interest to declare.

Authors' Contributions. Study design: Paulo Rabna, Christian Wejse, Peter Aaby, Jesper Eugen-Olsen; Study Implementation and data collection: Paulo Rabna, Christian Wejse, Ines Oliveira, Victor Francisco Gomes, Maya Bonde Haaland; Manuscript writing: Paulo Rabna, Andreas Andersen; Data analysis: Andreas Andersen, Paulo Rabna; Final manuscript review: all authors.

Funding Sources. The study was funded by the EU FP6 project number LSSP-CT-2005-012173. The funding Institution had no influence on design, and conduct of the study or on preparation, review and approval of the manuscript, neither on analysis, interpretation of the data or decision to publish.

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