# A Panel of Urine Derived Biomarkers to Identify and Distinguish Sepsis From Systemic Inflammatory Response Syndrome

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# Abstract

### Background

Sepsis is a fatal condition caused by infection. It is frequently difficult to distinguish sepsis from systemic inflammatory response syndrome (SIRS), often resulting in poor prognoses and the misuse of antibiotics. Hence, highly sensitive and specific biomarkers are needed to differentiate sepsis from SIRS.

### Methods

Urine samples were collected and segregated by group (sepsis group, SIRS group, healthy control group). iTRAQ was used to identify the differentially expressed protein among the three groups. The identified proteins were measured by ELISA from urine samples. Finally, all the acquired data was analyzed by SPSS.

### Results

The C-reactive protein, leucine-rich alpha glycoprotein-1 and serum amyloid A protein were differentially expressed among the three groups. The adjusted median concentrations of urinary C-reactive protein were 1337.6, 358.7, and 2.4 in the three groups, respectively. The urinary leucine-rich alpha glycoprotein-1 levels were 1614.4, 644.5, and 13.6 respectively. The levels of SAA were 6.3, 2.9, and 0.07, respectively. The sepsis group were higher than SIRS group ($P<0.001$) and the SIRS group were higher than healthy control group. The sensitivity was 0.906 and the specificity was 0.896 when combining the three biomarkers to distinguish sepsis from SIRS.

### Conclusion

The urinary C-reactive protein, urinary leucine-rich alpha glycoprotein-1 and urinary SAA has diagnostic value in cases of sepsis. Combining the three biomarkers would greatly improve the sensitivity and specificity of differential diagnoses between sepsis and SIRS.


# Introduction

Sepsis was first defined in 1992 [1] as a systemic inflammatory response syndrome (SIRS) caused by confirmed infection (Sepsis 1.0). The signs and symptoms of sepsis were expanded in Sepsis 2.0 [2]. Sepsis is now defined as organ dysfunction caused by systemic inflammatory response to pathogenic microorganisms, which can be fatal (Sepsis 3.0) [3]. In the last decade, the incidence rate of sepsis was 437 per 100,000 person-years and the incidence of severe sepsis (defined by Sepsis 2.0 [2]) was 270 per 100,000 person-years in developed countries [4]. Fleischmann et al. [4] also inferred that there were 31.5 million sepsis and 19.4 million severe sepsis cases, worldwide, with 5.3 million deaths annually. The case-fatality rate could reach up to 30% in sepsis, 50% in severe sepsis and 80% in cases of septic shock [5]. In addition, the prevalence of sepsis and contribution of sepsis to all-cause mortality rates have been increasing in the last several years [6]. Severe sepsis in elderly patients was an independent risk factor for

substantial and persistent new cognitive impairment and functional disability among survivors [7]. Delays in treatment and inappropriate antibiotic therapy would dramatically reduce the survival rates in septic shock [8, 9]. Hence, the early diagnosis of sepsis is particularly important. At present, the diagnosis of sepsis is difficult and complicated. Although there are clinical guidelines and many laboratory tests, e.g. C-reactive protein (CRP) test, procalcitonin (PCT) test, white blood cell (WBC) count, etc., to diagnose the sepsis, the specificity is unsatisfactory. Blood culture remains the conclusive evidence for the diagnosis of sepsis, but the sensitivity of blood culture is very low and blood culture is time-consuming, usually resulting in the delay of diagnosis. On the other hand, SIRS patients could be misdiagnosed with sepsis, which leads to antibiotic misuse and possible selection for drug resistance, because the symptoms and signs of SIRS are very similar to sepsis when the blood culture is negative. Biomarkers refer to measurable and quantifiable biological parameters which could be molecules, genes, proteins or other characteristics. There are hundreds of biomarkers, but useful biomarkers against sepsis are only a small fraction [10], e.g. CRP, PCT, serum amyloid A (SAA), triggering receptors expressed on myeloid cells-1 (TREM-1) and others. Biomarkers are a promising way to diagnose sepsis which will facilitate early and accurate diagnosis, forecast organ dysfunction and assist in defining the appropriate therapeutic plan [11]. Human urine contains thousands of proteins [12, 13] and extracellular vesicles [14] which could be good resources for biomarkers. Isobaric tags for relative and absolute quantification (iTRAQ) is a mass spectrometry (MS) based relative proteomic quantification method, utilizing the derivatization of primary amino groups in intact proteins and isobaric tags for different peptide fragments [15, 16]. iTRAQ can be used to screen the differentially expressed proteins among eight samples simultaneously and it is highly sensitive and specific [17]. In this study, we sought to identify new diagnostic biomarkers of sepsis in urine, utilizing iTRAQ, and verify the biomarkers using enzyme linked immunosorbent assays (ELISA). In addition, we explored the diagnostic value of the combined biomarkers through logistic regression in order to enhance the diagnostic sensitivity and specificity to sepsis and help clinicians determine the appropriate therapeutic strategy.

# Materials And Methods

Urine samples and data collection

This study was performed at the Second Affiliated Hospital of Chongqing Medical University. The procedures used in this study were in accordance with the 2008 Declaration of Helsinki and approved by the Ethics Committee of the Second Affiliated Hospital of Chongqing Medical University (Grant no. 201916). The urine samples and patient data came from the Adult Multidisciplinary Intensive Care Unit (ICU) and Infection department. All patients or their bailors were informed about the study procedures, risk and privacy policy and written consent was signed by each participant. Urine samples were collected from patients in the sepsis group, SIRS group and healthy control group. Consecutive patients with sepsis or SIRS were enrolled and the healthy control group were enrolled by recruiting. Sepsis inclusion criteria [3] were: (1) Suspected or proven infection, (2) Sequential [sepsis-related] Organ Failure Assessment (SOFA) score or 2 or more points. The specific criteria of the SOFA score are listed in Table 1 [3, 18] and the effectiveness of the SOFA was assessed [19]. SIRS inclusion criteria [1] were: (1)Temperature >38°C or

<36°C; (2) heart rate >90/min; (3) respiratory rate >20/min or $PaCO_2$ <32mmHg; (4) white blood cell count >12´$10^9$/L or <4´$10^9$/L or >10% immature bands in peripheral blood. The patients who were diagnosed with SIRS must have presented with two or more of the four criteria. Healthy control inclusion criteria were that the healthy volunteer who did not suffer from infection disease or take antibiotics. Exclusion criteria were: (1) immune deficiency, (2) autoimmune diseases, (3) refusal to take part in the study or refusal to provide signed, written consent. The urine samples were collected as soon as the clinical diagnosis was made and the interval time was more than four hours from the patients' last micturition. The urine was collected in 50 mL centrifuge tube directly from the bladder and no protease inhibitor was added to the samples. The collected samples were transported in an ice-water mixture. These samples were centrifuged at 2000g for 10min to separate the cellular or tissue debris. Finally, the samples were divided to five 2.0 ml Eppendorf tubes and stored at -80℃ until analysis [20]. The patient data were collected through the electronic medical system of the Second Affiliated Hospital of Chongqing Medical University.

| Table 1, Sequential [Sepsis-Related] Organ Failure Assessment Score [3, 18] | | | | | |
|---|---|---|---|---|---|
| Score<br><br>Criteria | 0 | 1 | 2 | 3 | 4 |
| $PaCO_2/FiO_2$ (mmHg) | ³400 | <400 | <300 | <200 | <100 |
| Platelets (´$10^9$/L) | ³150 | <150 | <100 | <50 | <20 |
| Bilirubin (mmmol/L) | <20 | 20-32 | 33-101 | 102-204 | >204 |
| MAP (mmHg) | ³70 | <70 | Dopamine <5 or<br><br>dobutamine (any dose) [a] | Dopamine 5.1-15<br><br>or epinephrine ≤0.1 or norepinephrine ≤0.1 [a] | Dopamine >15 or<br><br>epinephrine >0.1<br><br>or norepinephrine >0.1[a] |
| Glasgow Coma Scale<br><br>Score [21] | 15 | 13-14 | 10-13 | 6-9 | 6 |
| Creatinine (mmol/L) | <110 | 110-170 | 171-299 | 300-400 | 440 |
| Urine output (ml/d) | - | - | - | <500 | <200 |
| PaO2, partial pressure of oxygen; FIO2, fraction of inspired oxygen; MAP, mean arterial pressure; [a] Catecholamine doses are given as μg/(kg×min) for at least 1 hour. | | | | | |

Materials and measurement procedure

Protein extraction from urine and iTRAQ procedure

We collected 10 samples from each of the groups to perform a preliminary analysis of the differentially expressed proteins among the three groups. The samples were pooled by group (sepsis, SIRS, and healthy control) and the proteins were precipitated using two-fold volumes of cooled acetone (2h) [13]. Finally, the three samples were centrifuged at 2,5000g at 4℃ for 15 min and the supernatants were discarded. This procedure was repeated twice.

The precipitates were air-dried and dissolved at room temperature in an appropriate amount of lysis buffer using an ultrasonic homogenizer. Finally, the solutions were centrifuged at 2,5000g at 4℃ for 15

min and the supernatants were collected. The Bradford method was used to measure the protein concentrations.

An iTRAQ 8 Plex Reagent Kit (Applied Biosystems, Foster City, CA, USA) was used to label the protein. The prepared protein was precipitated, re-dissolved, alkylated, cysteine-blocked and digested following the iTRAQ Kit instructions and the study by Wisniewski [22]. The Sepsis group was labelled with tag 113, the SIRS group was labelled with tag 119 and the healthy control group was labelled with tag 121. The labelled peptide solutions were pooled and freeze-dried under vacuum prior to further analysis.

The peptides were fractioned as previously described [23, 24]. The prepared peptides were analyzed on the TripleTOF 5600 system coupled to an Eksigent NanoLC- 2D system (AB Sciex, Framingham, MA, USA) and each of the samples was analyzed twice. The data was processed by ProteinPilot V2.0 (Applied Biosystems) and searched against the UniProt human proteome database (https://www.uniprot.org/). The proteins having Unused Protscore >1.3, fold change >2 and P-value <0.05 were defined as differentially expressed.

ELISA measurement procedure

We employed the Human C Reactive Protein ELISA Kit (ab9995, Abcam, UK), Human LRG1 ELISA Kit (NBP2-60577, Novus Biologicals, Centennial, CO, USA), Human Serum Amyloid A ELISA Kit (KT-547, Kamiya Biomedical Company, Seattle, WA, USA) to measure the concentrations of target proteins. Urine samples from each patient were measured by ELISA and the measurement protocol was performed according to the instructions of the three respective ELISA kits. The specifics of each ELISA kit are listed in Table 2. The duplicate standards or samples were set and mean absorbance of duplicate wells was calculated. The concentration of urinary proteins (CRP, LRG1, SAA) was normalized to urinary creatinine (u-Cr) and expressed as protein/u-Cr in μg/mmol to adjust for individual differences and potential kidney injury [25, 26]. All statistical analyses were performed using Microsoft Excel 2019 (Microsoft Corporation, Redmond, WA, USA) and SPSS 25 (International Business Machines Corporation, Armonk, NY, USA).

Table 2. the characteristics of ELISA kit

| Trait | Sensitivity | Working Range | Coefficient of Variation (CV, %) | |
|---|---|---|---|---|
| Reagent | | | Intra-Assay | Inter-Assay |
| CRP ELISA Kit | 2 pg/ml | 34.29-2,5000 pg/ml | <10 | <12 |
| LRG1 ELISA Kit | 0.17 ng/ml | 0.313-20 ng/ml | <3.1 | <9.4 |
| SAA ELISA Kit | 0.50 ng/ml | 1.56-100 ng/ml | <8.5 | <11 |

# Results

Subjects characteristics

There were a total of 151 subjects included in our study: 53 sepsis patients, 48 SIRS patients and 50 healthy volunteers. The average ages of the three groups were 58.4, 62.0 and 51.0 years, respectively. The percentage of males was 64.2%, 75%, and 56%, respectively. In the sepsis group, there were 24 patients whose blood cultures were positive. The detected bacteria consisted of *Escherichia coli* (10, 41.7%), *Staphylococcus aureus* (6, 25%), *Enterococcus faecium* (3, 12.5%), *Acinetobacter baumannii* (2, 8.3%), *Streptococcus* (1, 4.2%), *Lactobacillus brevis* (1, 4.2%) and *Klebsiella pneumoniae* (1, 4.2%). There were no statistically significant differences in the distributions of age or gender among the three groups. The distribution of WBC count and neutrophil percentage were statistically similar and could not distinguish the sepsis and SIRS patients in this study. In addition, the sepsis patients presented higher urinary creatinine levels and pulse rates compared to the healthy control group. Patient data is summarized in Table 3.

| Table 3. Subject characteristics and quantitative analysis | | | | |
|---|---|---|---|---|
| | Sepsis (n=53) | SIRS (n=48) | Healthy (n=50) | P value |
| Age | 62.0 (47.0, 73.0) | 65.0 (49.8, 71.3) | 61.0 (48.5, 70.5) | NS[a, b, c] |
| Males, n (%) | 34 (64.2) | 36 (75) | 28 (56) | NS* |
| WBC (´$10^9$/L) | 10.3 (6.9, 13.1) | 11.5 (8.0, 14.0) | N | NS[a] |
| Neutrophil (%) | 86.7 (82.5, 90.2) | 86.0 (81.0, 89.0) | N | NS[a] |
| s-CRP (mg/L) | 149.5 (105.1, 183.9) | 59.7 (21.1, 80.6) | N | P<0.001[a] |
| s-Cr (μmol/L) | 78.5 (53.9, 137.2) | 55.7 (45.0, 71.6) | N | P< 0.001[a] |
| u-Cr (mmol/L) | 4.9 (3.5, 7.8) | 4.4 (3.6, 5.5) | 4.4 (3.3, 5.3) | NS[a, c], P=0.041[b] |
| T (℃) | 38.0 (37.2, 38.8) | 38.0 (36.4, 41.0) | N | NS[a] |
| R (/min) | 20 (16, 22) | 21 (14, 32) | N | NS[a] |
| P (/min) | 110 (90, 124) | 100 (74, 140) | N | P=0.03[a] |
| uCRP (μg/L) | 6738.2 (4043.5, 10393.9) | 2050.8 (637.6, 3283.5) | 10.6 (8.0, 13.1) | P<0.001[a, b, c] |
| uLRG1 (μg/L) | 7701.8 (4835.6, 16180.7) | 2724.6 (1945.3, 4676.8) | 55.5 (52.5, 61.5) | P<0.001[a, b, c] |
| uSAA (μg/L) | 29.0 (23.8, 44.9) | 14.3 (8.8, 18.5) | 0.32 (0.30, 0.38) | P<0.001[a, b, c] |
| uCRP/u-Cr (μg/mmol) | 1337.6 (773.8, 1822.3) | 358.7 (149.8, 786.5) | 2.4 (1.8, 3.3) | P<0.001[a, b, c] |
| uLRG1/u-Cr (μg/mmol) | 1614.4 (1197.3, 2145.1) | 644.5 (469.5, 1077.7) | 13.6 (10.7, 18.6) | P<0.001[a, b, c] |
| uSAA/u-Cr (μg/mmol) | 6.3 (4.7, 8.8) | 2.9 (1.7, 4.1) | 0.079 (0.061, 0.108) | P<0.001[a, b, c] |
| WBC, white blood cell; s-CRP, serum C-reactive protein; s-Cr, serum creatinine; u-Cr, urinary creatine; uCRP, urinary CRP; uLRG1, urinary LRG1; uSAA, urinary SAA; T, body temperature; R, respiratory rate; P, pulse rate; N, none data; Variables are expressed as median (25% percentiles, 75% percentiles); Mann-Whitney U test or chi-square test were performed to test the statistical significance between different groups; a, tested by Mann-Whitney U test between the sepsis group and SIRS group; b, tested by Mann-Whitney U test between the sepsis group and healthy control group; c, tested by Mann-Whitney U test between SIRS group and healthy control group; *, tested by chi-square test; Significance level is P<0.05. | | | | |

iTRAQ identification of differentially expressed proteins

iTRAQ identified 11278 and 14904 proteins in duplicate analyses. There were 1970 and 1985 proteins with unused ProtScore > 1.3. Among these proteins, there were 114 and 118 with fold change> 2 between the sepsis group and healthy control group. There were 69 proteins which were detected in the both iTRAQ procedures. After eliminating the proteins with P values > 0.05, there were 45 proteins meeting the screening criteria. The flow chart is presented in Figure 1. CRP, LRG1, SAA were included in the 45 proteins. These three biomarkers were further analyzed by ELISA and their iTRAQ information are listed in Table 4.

Table 4. iTRAQ information of identified biomarkers

| | CRP | LRG1 | SAA |
|---|---|---|---|
| Accession | sp\|P02741\|CRP_HUMAN | sp\|P02750\|A2GL_HUMAN | tr\|D3DQX7\|D3DQX7_HUMAN |
| Unused | 115.64 | 59.08 | 83.08 |
| 113:119 | 2.68 | 3.49 | 2.75 |
| P value (113:119) | 1.81E-12 | 0.000162 | 6.47E-5 |
| 119:121 | 6.93 | 4.14 | 7.26 |
| P value (119:121) | 3.81E-11 | 2.96E-7 | 5.88E-9 |

Quantitative and statistical analysis

The concentrations of uCRP, uLRG1 and uSAA are shown in Table 3. Considering the variability of glomerular filtration rate (GFR), 24-hours urinary volume and glomerular filtration barrier among the different subjects, the concentrations normalized to urinary creatinine levels were also listed and expressed as uCRP/u-Cr, uLRG1/u-Cr and uSAA/u-Cr in μg/mmol and were used for further statistical analyses. The normality of acquired data was tested and we found that not all the data followed a Gaussian distribution. Although efforts were made to transform the data to a Gaussian distribution by converting initial data to the square root or logarithm, the data did not conform. So the data were presented as medians (25 percentile%, 75% percentile) and non-parametric tests were used to analyze the statistical characteristics. The Spearman's rank correlation coefficient was calculated (Table 5). The serum CRP was strongly correlation with the urine CRP, which may indicate that the urinary CRP came from the blood. In addition, the three biomarkers (uCRP, uLRG1, uSAA) were pairwise correlated, which may indicate that they had similar status during the infection process. The median values of sepsis patients were highest, the SIRS patients were second and the healthy control group were lowest ($P < 0.001$).

| Table 5. Spearman's rank correlation coefficient | | | | |
|---|---|---|---|---|
| | uCRP | uLRG1 | uSAA | sCRP |
| uCRP | 1 | | | |
| uLRG1 | 0.822[a] | 1 | | |
| uSAA | 0.853[a] | 0.829[a] | 1 | |
| sCRP | 0.846[a] | 0.493[a] | 0.493[a] | 1 |
| a. The P value is <0.001 | | | | |

When these biomarkers were adjusted to urine creatinine, the same conclusion could be inferred too and their values are presented in Figure 2. For the adjusted concentrations and sCRP, receiver operating characteristic curves (ROC) were drawn by SPSS 25 (Figure 3). The area under the curve values were 0.878 (uCRP/u-Cr), 0.874 (uLRG1/u-Cr), and 0.849 (uSAA/u-Cr), 0.891 (sCRP). The Youden index (sensitivity + specificity – 1) was used to determine the best cut-off value. The sensitivity, specificity, cut-off value and area under the curve values are listed in Table 6. The sCRP had the highest diagnostic efficiency. The diagnostic difference was small among uCRP/u-Cr, uLRG1/u-Cr and uSAA/u-Cr. The adjusted concentrations (uCRP/u-Cr, uLRG1/u-Cr and uSAA/u-Cr) of the sepsis and SIRS groups were transformed to binary variables based on whether they were greater than the cut-off value and further analyzed by logistic regression. Forward stepwise (likelihood ratio) method was employed to process the data. The Omnibus test was used to verify the statistical significance (P<0.001) and Hosmer and Lemeshow test showed the model had taken full advantage of the acquired data (P=0.712). The regression coefficient and P values are listed in Table 7. According to the regression results, all three biomarkers were included in the regression model. If the patients' parameters exceeded the cut-off value, the corresponding risk of sepsis would increase 9.913-fold (uCRP/u-Cr), 15.936-fold (uLRG1/u-Cr) and 12.793-fold (uSAA/u-Cr) compared to SIRS patients. The risk score of sepsis is Logit (P) $=2.294*X_1+2.734*X_2+2.549*X_3-3.714$ ($X_1$=1, if uCRP/u-Cr >746.1 μg/mmol; $X_1$=0, if uCRP/u-Cr>746.1 μg/mmol; $X_2$=1, if uLRG1/u-Cr >1174.8μg/mmol; $X_2$=0, if uLRG1/u-Cr <1174.8μg/mmol; $X_3$=1, if uSAA/u-Cr >4.4μg/mmol; $X_3$=0, if uSAA/u-Cr <4.4μg/mmol). According to the Logit(P), the prediction probabilities were calculated by $P=e^{Logit(P)}/(1+e^{Logit(P)})$ and its ROC was also drawn (Figure 4). The area under the curve was 0.937. The cut-off of Logit(P) was 0.514. Its sensitivity and specificity were 0.906 and 0.896, respectively. This indicated that sensitivity and specificity would be greatly enhanced when diagnosis was made by combining the panel of biomarkers.

| Table 6. The diagnostic value of identified biomarkers | | | | | |
|---|---|---|---|---|---|
| | Area | Cut-off (μg/mmol) | Sensitivity | Specificity | Youden index |
| uCRP/u-Cr | 0.878 | 746.1 | 0.849 | 0.729 | 0.578 |
| uLRG1/u-Cr | 0.874 | 1174.8 | 0.774 | 0.854 | 0.628 |
| uSAA/u-Cr | 0.849 | 4.4 | 0.792 | 0.792 | 0.584 |
| sCRP | 0.891 | 87.76 | 0.868 | 0.833 | 0.701 |

| Table 7. Variables in the equation | | | | | |
|---|---|---|---|---|---|
| | B | Sig. | Exp(B) | 95% C.I. for Exp(B) | |
| | | | | Lower | Upper |
| uCRP/u-Cr | 2.294 | 0.001 | 9.913 | 2.608 | 37.677 |
| uLRG1/u-Cr | 2.734 | <0.001 | 15.396 | 3.307 | 49.489 |
| uSAA/u-Cr | 2.549 | <0.001 | 12.793 | 3.926 | 60.380 |
| Constant | -3.714 | <0.001 | 0.024 | - | - |
| B, Regression coefficient; Sig., Statistical significance; Exp(B), equal to Odds ratios; 95% C.I. refers to 95% confidence interval. | | | | | |

# Discussion

Because the symptoms of sepsis and SIRS are quite similar, it is frequently difficult to distinguish a septic infection from SIRS. After Sepsis 3.0 was issued, the situation become more difficult, in part because patients suffering from infection-related SIRS, but without organ dysfunction, can be confused with patients having SIRS without sepsis. Biomarkers, and especially acute-phase proteins, could be key to distinguishing sepsis from SIRS, but their specificity has been unsatisfactory, making it necessary to combine multiple biomarkers in order to enhance overall specificity [27, 28]. The three biomarkers identified in the present study were all acute phase reaction proteins [29-32]. CRP was identified in the 1930s and is synthesized in the liver and released in response to infection, trauma and immune disorders. Mean sCRP levels were 98mg/L in sepsis patients and 70mg/L in SIRS patients [33]. Most recently, bacterial infection is suspected when sCRP is greater than 100mg/L in the clinical context. Conversely, absence of severe bacterial infection is indicated when sCRP is less than 20mg/L [30]. In the present study, we first verified the existence of CRP in urine and uCRP/u-Cr was strongly relation to sCRRP. Although the diagnostic efficiency of uCRP/u-Cr was slightly lower than sCRP, uCRP/uCr could still be a good substitution for sCRP considering the convenience of specimen collection, strong relation and its

noninvasiveness. Leucine rich α-2 glycoprotein 1 (LRG1), first identified in 1977 [34], is synthesized by hepatocytes, stored in the neutrophils [35] or myeloid cells [36], and released to serum when the body is in acute phase stimulation by bacterial or viral infection [37]. LRG1 belongs to the leucine rich repeat (LRR) protein family and plays a role in protein interactions, innate immunity, platelet aggregation and angiogenesis. LRG1 is usually used as a biomarker of tumors, appendicitis, diabetes complications, and inflammatory disease [38-41]. According to our measurement results, uLRG1/u-Cr of sepsis patient was obviously higher in sepsis patients than in either SIRS patients or healthy volunteers ($P<0.001$), indicating that urinary LRG1 is a promising biomarker in diagnosing sepsis and distinguishing sepsis from SIRS. Considering the lower sensitivity (0.774) and higher specificity (0.854), LRG1 would be a good choice to combine with other biomarkers. Serum amyloid A protein is also a well-known acute phase protein produced by liver. Its concentration is very low (1 μg/ml) in healthy people, but can increase dramatically (100-fold or more) when human body is in acute phase [42]. Human SAA consists of 4 isotypes (SAA1, SAA2, SAA3,SAA4) [43], but sequence identity of SAA1 and SAA2 is more than 93% and SAA1 is the main isotype in serum [44]. The SAA ELISA Kits we employed in the current study were not sensitive to the SAA isotypes, and the concentration represented the concentration of total SAA. SAA is regulated by cytokines (Interleukin-1β, Interleukin-6, Tumor necrosis factor, etc.) and regulate cytokines (Interleukin-23, Interleukin-33, Interleukin-10, etc.) which is summarized as the cytokine-SAA-chemokine network [45]. Considering its interaction with cytokines, SAA was deemed to take part in inflammatory diseases, angiogenesis and tumor growth as do other acute phase proteins [46-48]. In our study, the sensitivity and specificity of uSAA/u-Cr were between the uCRP/u-Cr and uLRG1/u-Cr. It was also the first time the value of uSAA to sepsis diagnosis and differential diagnosis was verified.

Although the three biomarkers were normalized to urinary creatinine in the statistical process, adjusting concentration had only a small influence on the statistical results and no influence on the final conclusion. When the panel of biomarkers were combined, the sensitivity to sepsis was increased to 0.906 and specificity was increased to 0.896, which greatly exceeded any single biomarker. The area under ROC curve was 0.937, which indicated favorable diagnostic efficiency. Serum CRP data was acquired through the medical electronic system, but the serum LRG1 and SAA were not available by routine examination in the Second Affiliated Hospital of Chongqing Medical University. Hence serum LRG1 and SAA data was lacking in our study. The correlation between serum LRG1 and urinary LRG1 was not verified, and neither was the correlation of serum SAA and urinary LRG1. According to the correlation between serum CRP and urinary CRP, we inferred that urine CRP came from serum CRP. Considering the similar mechanism of protein excretion in kidney, we hypothesized urinary LRG1 and SAA also came from blood. There were only 101 patients included in our study, and it would be necessary to recruit more patients to further verify the efficiency of the combined biomarkers. In addition, the diagnostic value of LRG1 to sepsis was not fully investigated, and additional research is necessary to further understand the diagnostic significance of LRG1.

# Conclusions

C-reactive protein, serum amyloid A protein and leucine-rich alpha glycoprotein-1 exist in the urine. The urinary C-reactive protein comes from blood and may be a substitution of serum C-reactive protein in sepsis diagnosis. Urine SAA and leucine-rich alpha glycoprotein-1 were also favorable biomarkers in sepsis diagnosis. The sensitivity and specificity of differential diagnosis between sepsis and systemic inflammatory response syndrome could be greatly improved when the three biomarkers are combined.

# Abbreviations

SIRS, systemic inflammatory response syndrome

CRP, C-reactive protein

uCRP, urinary CRP

PCT, Procalcitonin

SAA, Serum amyloid A protein

uSAA, urinary SAA

TREM-1, Triggering receptors expressed on myeloid cells-1

iTRAQ, Isobaric tags for relative and absolute quantification

ELISA, Enzyme linked immunosorbent assay

ICU, Intensive care unit

SOFA, Sequential [Sepsis-related] organ failure assessment

LRG1, Leucine-rich alpha glycoprotein-1

uLRG1, Urinary LRG1

u-Cr, Urinary creatinine

CV, Coefficient of variation

SPSS, Statistical product and service solutions

GFR, Glomerular filtration rate

ROC, Receiver operating characteristic curve

LRR, Leucine rich repeat

# Declarations

Authorship statement

XHW and HDH conceived and designed the study and are both guarantors of the study. YT and AZ had collected the urine sample and patients' data. YT and WYZ performed the iTRAQ and ELISA procedures. JH was responsible for statistical analysis. YT wrote the paper. HR and YXY edited the clinical theses. All the authors have approved the final manuscript and declare that there exist no conflicts among them.

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# Figures

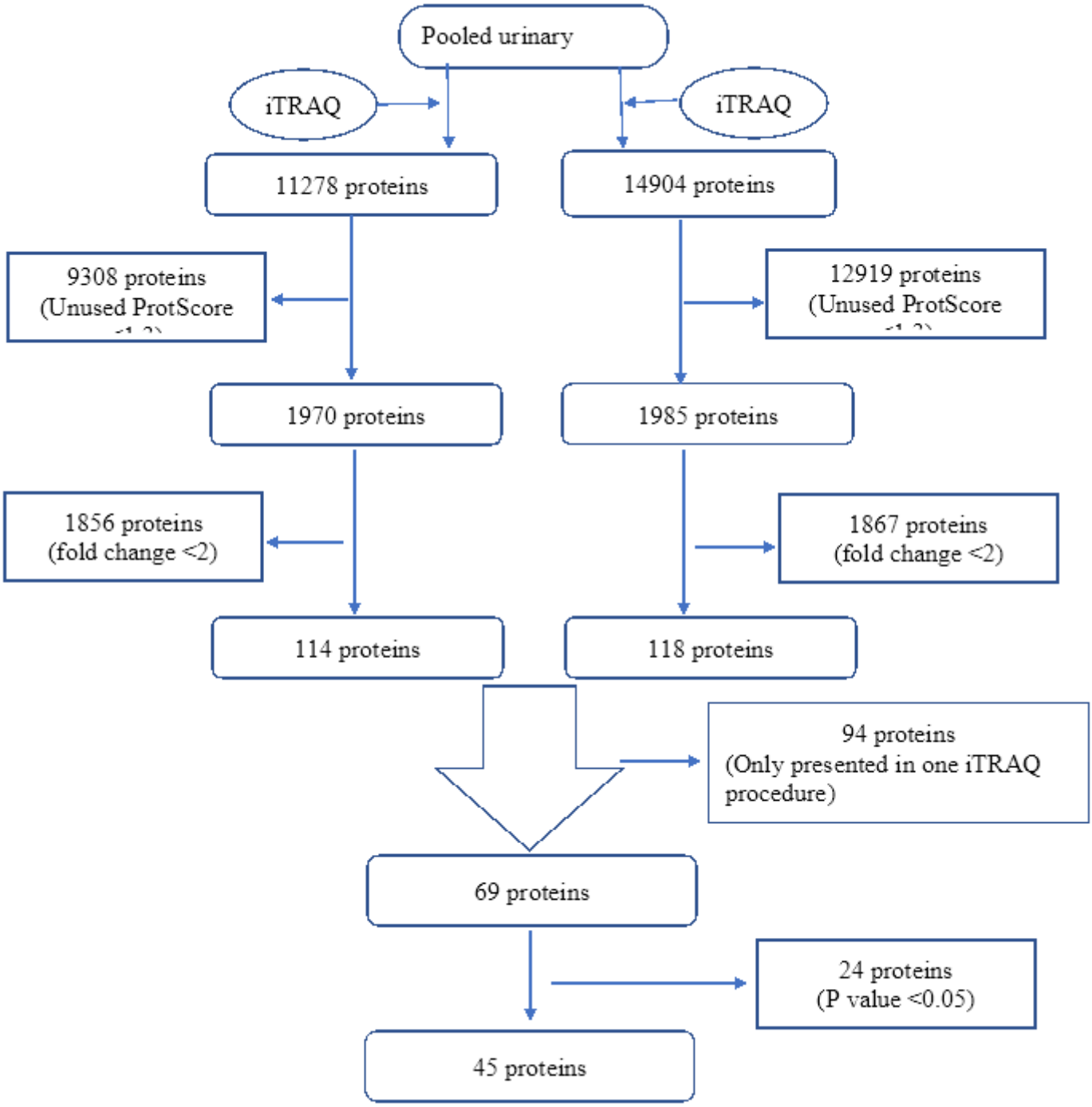
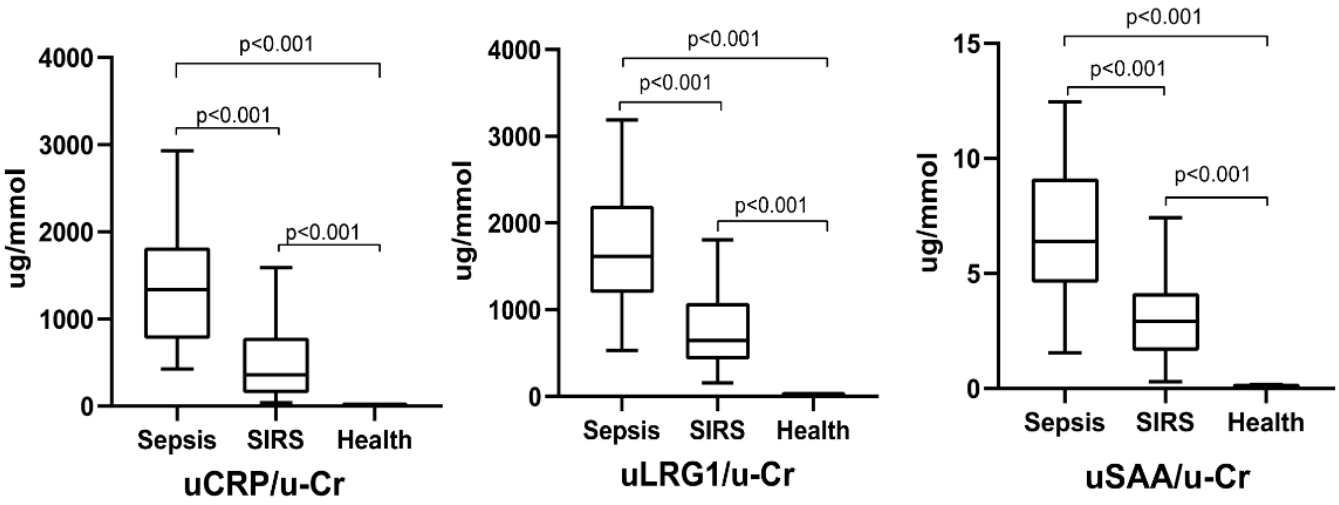


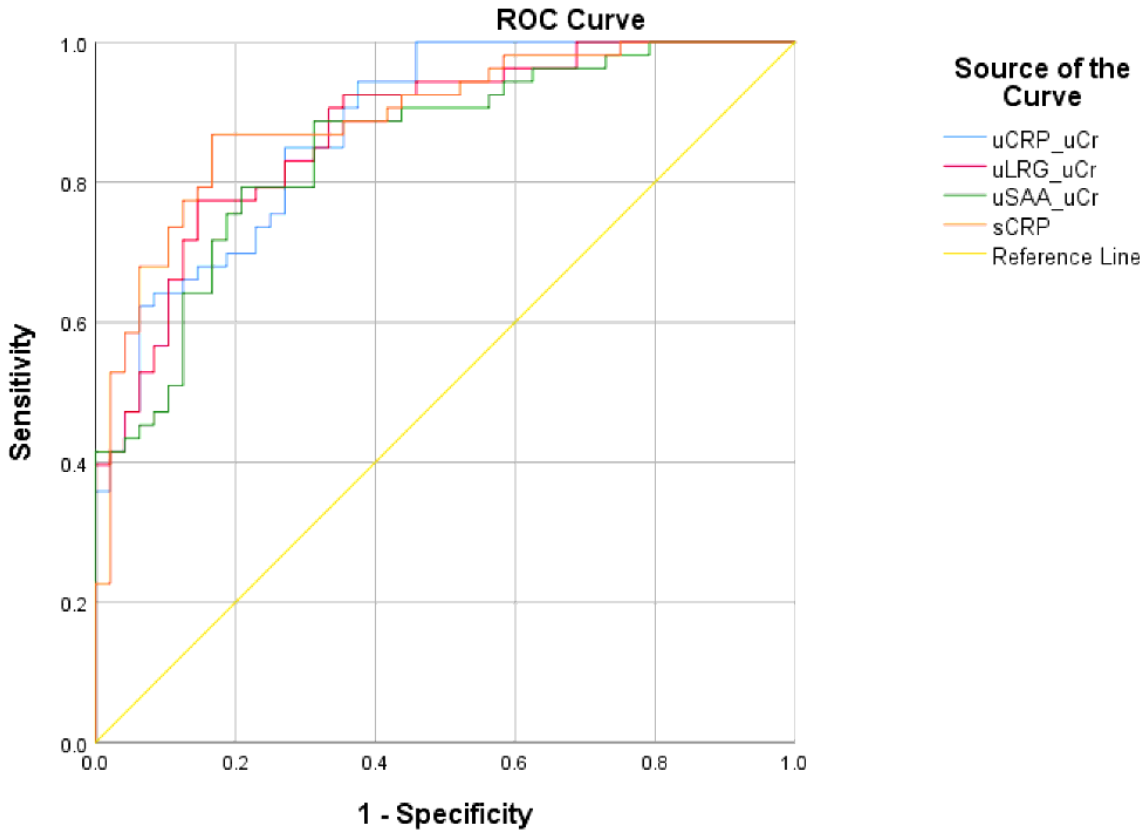

**Figure 1**

Flow chart of protein identification




## Figure 2

Box-plot of adjusted concentration




## Figure 3

The ROC curve of adjusted concentration and sCRP

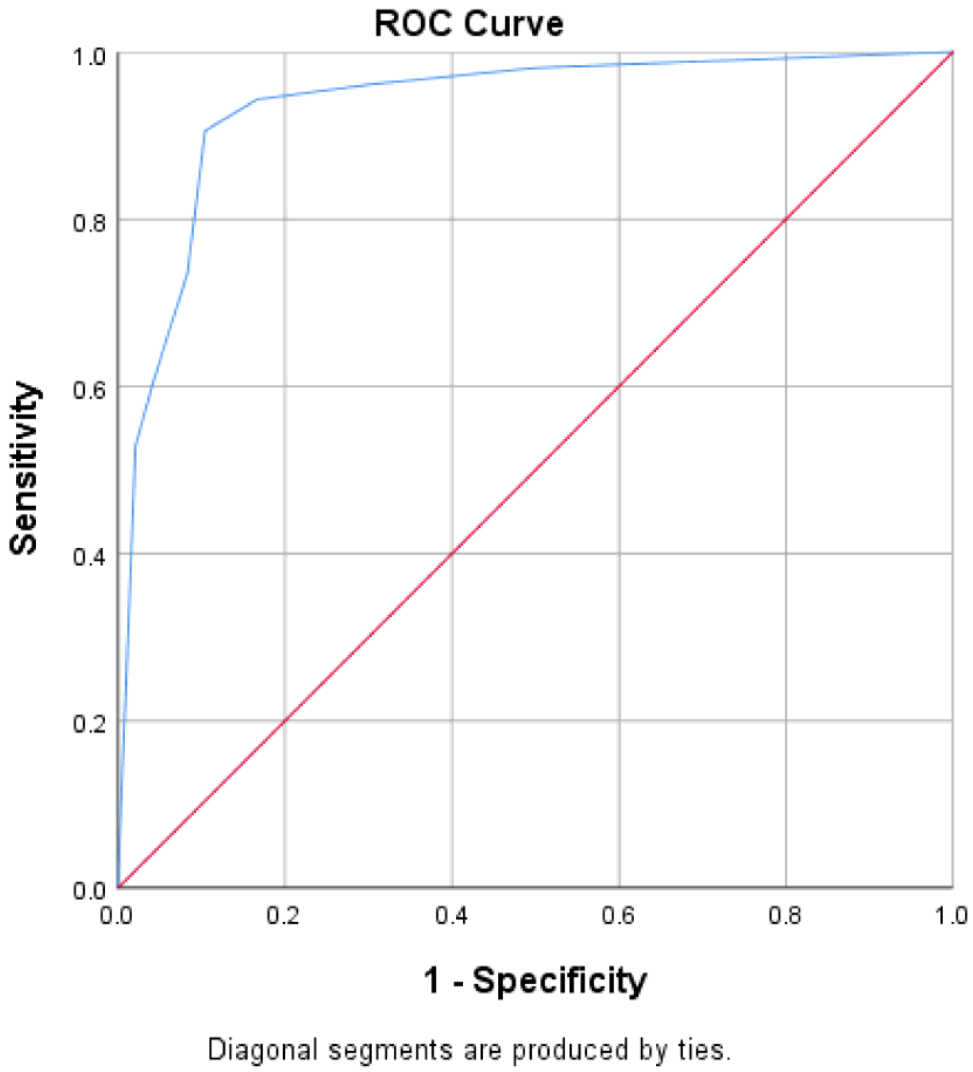



**Figure 4**

ROC Curve of prediction probalilities