Rapid and sensitive detection of L-FABP for prediction and diagnosis of acute kidney injury in critically ill patients by chemiluminescent immunoassay

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Abstract

Background: Acute kidney injury (AKI) was a common clinical complication among critically ill patients in Intensive Care Unit with high morbidity and mortality. Human liver fatty acid-binding protein (L-FABP) as a renal tubular injury biomarker was considered a predictor of AKI; however, high-throughput and sensitive detection methods were still urgently needed. We constructed a sensitive and rapid detection method for detecting L-FABP and for exploring the clinical application of L-FABP as a predictor for AKI.

Methods: We developed an automated detection method of chemiluminescent immunoassay to measure L-FABP and evaluated the analytical performance of the new methodology including analytical selectivity, analytical sensitivity, linear range, the minimum limit of detection (LOD), repeatability, and accuracy. One hundred patients were enrolled in this study to explore the predictive and diagnostic ability for AKI.

Results: The chemiluminescent immune-based L-FABP assay had outstanding analytical sensitivity including the detection limit of 0.88 ng/ml, and a wide linear range of 2 ng/ml to 160 ng/ml. It also exhibited excellent repeatability with intra-analysis CVs of 8.73%, 4.72%, and 3.79%, respectively, and the inter-analysis CVs of 13.47%, 7.28%, and 5.94%, respectively. The recovery rate assay exhibited a good accuracy with three L-FABP concentration of 99.76%, 102.27%, and 96.92%, respectively. The reference interval of L-FABP was between 0.88 ng/ml and 5.98ng/ml. The evaluation of predictive and diagnostic performance showed that higher concentration of L-FABP indicated higher risk of AKI occurrence and disease progression.

Conclusions: The clinical application of rapid and sensitive detection method of L-FABP based on the newly developed chemiluminescent immunoassay could offer benefits for patients. L-FABP was a potentially predictive and diagnostic biomarker for AKI.
1 | INTRODUCTION

Acute kidney injury (AKI) is a common comorbidity of critically ill patients, which refers to a clinical syndrome characterized by a rapid decrease in renal excretory function, with the accumulation of products of nitrogen metabolism such as urea and creatinine and other clinical unmeasured waste products. According to research statistics, 7% of hospitalized patients could have AKI, and the morbidity of AKI in the intensive care unit (ICU) was as high as 25%. However, there were no effective treatments currently except for supportive renal replacement therapy such as dialysis. AKI is a common and important diagnostic and therapeutic challenge for clinicians. Although with the deepening of AKI research and new technologies and treatments are also emerging, such problems continue to appear. At present, the main diagnostic biomarkers of clinical reuse were serum creatinine (Scr) and urine volume. These two biomarkers had been used for many years. When kidney injury occurred, Scr due to the kidney’s own compensation mechanism could not reflect the change of kidney very sensitively and would lag behind the progress of the disease. The urine volume would be affected by clinical drugs, diuretics, and so on, which also could not truly reflect the progress of the kidney disease. The novel diagnostic technologies and biomarkers may help with early diagnosis. Therefore, it was urgent and significant to find early sensitive and specific diagnostic biomarkers to improve the long-term survival rate and postoperative recovery of patients with AKI.

Human liver fatty acid-binding protein (L-FABP) was located as a cytoplasmic protein in proximal renal tubular cells of the human kidney, renal L-FABP expression was upregulated, and urinary excretion of renal L-FABP was increased by various stressors, such as urinary protein, hyperglycemia, tubular ischemia, toxins, and salt-sensitive hypertension, which led to the progression of kidney disease. L-FABP could be checked to indicate whether there was kidney damage. A number of studies had shown that L-FABP had a good diagnostic value in kidney diseases including AKI, chronic kidney disease, and diabetic nephropathy. However, few indicated whether L-FABP had the predictive value of AKI. So, it was vital to explore the predictive value of L-FABP for AKI. Early prediction of AKI was important for clinical practice. The aim of this research was to assess whether L-FABP could be a predictor for AKI.

Advances in enzyme-linked immunosorbent assay (ELISA) technology have enabled the high-throughput detection of L-FABP, making it possible for analyzing in many studies conveniently. However, common ELISA-based methods cannot satisfy the rapid detection requirements. The sufficient sensitivity and specificity of methodology are required for complicated procedures. Therefore, based on the platform of our laboratory, we constructed the new methodology of chemiluminescent immunoassay for detecting L-FABP and explored the predictive value of L-FABP for AKI in critically ill patients.

2 | MATERIALS AND METHODS

2.1 | Apparatus and reagents

A photomultiplier instrument for chemiluminescent signal detection was developed by our laboratory. Chemiluminescent immunoassay instrument (Robust i1000) was provided by Hangzhou AiKang company. The incubation procedures were carried out using a constant temperature incubator. A magnetic separation device was purchased from Thermo Fisher Scientific (Invitrogen). Human liver fatty acid-binding protein (L-FABP) in its pure form used in this study was purchased from Sino biological (Sino Biological Inc.). Rabbit anti-human L-FABP polyclone antibody pair including detection and capture antibody was provided by Hangzhou Diag Biotechnology Co., Ltd. (China). Streptavidin-modified paramagnetic particles were purchased from Thermo Fisher Scientific (Invitrogen). N-hydroxysuccinimide biotin, acridinium ester, bovine serum albumin (BSA), phosphate-buffered saline (PBS), Tween-20, dimethylsulfoxide (DMSO), and anhydrous dimethyl formamide (DMF) were purchased from Sigma-Aldrich Ltd (USA). We prepared hydrogen peroxide and sodium hydroxide reagent. Washing buffer solution was made by PBS containing 1 mg/ml Tween-20 and 1 mg/ml BSA. Double-distilled water was prepared using water purified with an ultrapure water system (Zhejiang university second affiliated hospital). All other chemicals were standard commercial products of analytical reagent grade.

2.2 | Study design and participants

The inclusion criteria of this study was according to Kidney Disease Improving Global Outcomes (KDIGO) criteria patients were not diagnosed AKI when ICU admission. The exclusion criteria were patients having chronic renal diseases or patients who had undergone renal replacement such as dialysis or kidney transplant. The exit criterion was patients stayed in ICU less than 2 days. According to the inclusion and exclusion criteria, there were 112 patients admitted to the ICU of the Second Affiliated Hospital of Zhejiang University School of Medicine between December 2018 and November 2019. Excluding 12 patients who stayed in ICU less than 2 days. Finally, 100 patients were included in the study. AKI was defined according to the KDIGO guidelines as renal function was suddenly decreased within 48 h and Scr increased at least 0.3 mg/dl, or Scr increased more than 1.5 times higher than baseline within 7 days, or urine volume less than 0.5 ml/Kg per hour for 6 hours. AKI was classified as stage 1 (Cr at peak was 1.5 to 1.9 times baseline), stage 2 (Cr at peak was 2 to 2.9 times baseline), and stage 3 (Cr at peak was 3 times baseline or greater). Ethical approval for this study was obtained from the research ethical committees, Zhejiang University School.
of Medicine Second Affiliated Hospital. Blood samples were collected for all participants after they were admitted into ICU. Samples were collected every 24 h for 7 days. If patients were stayed in ICU less than 7 days, samples were stopped when out of ICU. All blood samples were centrifugated at 3000 rpm for 15 min to obtain plasma samples. Scr was measured by creatine oxidase method with Beckman Coulter AU5800 and L-FABP was measured by chemiluminescent immunoassay with Robust i1000. Plasma samples were stored for a maximum of 2 h at 4°C before analysis. For L-FABP assay, all measurements were repeated twice.

### 2.3 | Preparation of Biotin-labeled antihuman L-FABP antibody

Biotin-labeled antibody was prepared through the reaction between N-hydroxysuccinimide biotin and antihuman L-FABP antibody. The antibody solution was diluted with half volume of 0.1 M NaCl/0.1 mol/L sodium tetraborate (PH: 8.5). The N-hydroxysuccinimide biotin was dissolved in DMSO solution which the concentration was 2.8 mg/L. Then, the antibody solution and excess of the N-hydroxysuccinimide biotin with either 5:1, 10:1, 15:1, 20:1, or 25:1 molar were mixed with magnetically stirring at room temperature for an hour. Ultimately, the biotin-labeled antibodies were separated from the free biotin using sephadex G-75 chromatography column. The purified biotin-labeled antibody solution containing 0.2 mg/ml sodium azide and 2.5 mg/ml BSA was stored at −20°C.

### 2.4 | Preparation of acridinium ester-labeled antihuman L-FABP antibody

First, antihuman L-FABP antibody solution was diluted to 0.25 mg/ml by 0.1 M PBS (PH:7.4). Then, the antibody solution was reacted with 10 μl acridinium ester solution (0.5 mmol/L) previously dissolved in anhydrous DMF. The mixed solution was reacted at room temperature for half an hour. The acridinium ester-labeled antibody solution was also purified by sephadex G-75 chromatography column. The purified antibody solution containing 0.2 mg/ml sodium azide and 2.5 mg/ml BSA was stored at −20°C.

### 2.5 | Chemiluminescent immunoassay for detecting L-FABP

L-FABP was detected using a newly developed methodology of paramagnetic particle-based sandwich chemiluminescent immunoassay, which specifically recognized L-FABP. As is shown in Figure 1, 50 μl biotin labeled antibody (bio-Ab, capture antibody) 0.8 μg/ml in 50 mg/ml BSA-PBS solution, 50 μl acridinium ester labeled antibody (AE-Ab, detection antibody) 0.8 μg/ml in 50 mg/ml BSA-PBS solution and 10 μl sample solution which contained L-FABP antibody were stepwise mixed into microfuge tubes, followed by incubation at 37°C for 45 min. Afterward, 50 μl solution containing avidin-coated paramagnetic particles was added to mixtures in order to facilitate magnetic separation after bio-Ab-L-FABP-AE-Ab sandwich immunocomplexes were formed. After another 10 min for incubation at 37°C, an external magnetic field was used for removing uncombined paramagnetic particles. Washing buffer (600 μl) was added into the reaction cuvette and repeatedly washed three times. Then, 200 μl of H2O2 (trigger solution) was added to the mixtures for chemiluminescent signal excitation. Samples were analyzed by automated chemiluminescent apparatus. Before analyzing the samples, the standard curve of L-FABP was constructed by the standard materials of L-FABP. All samples were tested twice.

### 2.6 | Statistical analysis

The research data were analyzed by SPSS20.0, Graphpad prism 7.0, and R software 3.6.2. Continuous variables were presented as means (SD) when data satisfied normally distributed or as medians.
with skewed distribution. Categorical variables were presented as percentages. Continuous variables which conformed to the normal distribution were used a student's t-test for statistical differences, and which conformed to the skewed distribution were used Mann Whitney U test for statistical differences. Categorical variables were used Chi-square test. \( p < 0.05 \) was considered statistically significant. Receiver operating characteristic curve (ROC) was used for assessing the predictive value of occurrence risk of AKI; logistic regression was used for establishing models, which assessed the risk by measuring biomarkers before AKI happened. The rms package was used for assessing the relationship between the change of L-FABP concentration and the risk of AKI with R software 3.6.2 (https://cran.rstudio.com/).

3 | RESULTS

3.1 | Baseline characteristics of all participants

The clinical characteristics of the participants in this study were shown in Table 1. At last, 100 patients were recruited for this study. Among the patients, 15 patients were diagnosed with AKI and 85 patients with non-AKI. According to KDIGO criteria, there were 10 patients with AKI stage I, 3 patients with AKI stage II, and 2 patients with AKI stage III (Figure 2). The incidence of AKI in ICU was 15%. The baseline characteristics were analyzed between AKI cohort and non-AKI cohort. There were no significant differences in gender, age, BMI, hypertension, diabetes, smoking, drinking, K, Na, Cl, PLT, WBC, INR, and baseline creatinine.

3.2 | Screening the optimal reaction conditions

First, we evaluated the change trend of chemiluminescent efficiency due to different antibody concentrations, and then explored the optimal concentration of acridinium ester-labeled antihuman L-FABP antibody and biotin-labeled antihuman L-FABP antibody for L-FABP measurements. The concentration of the antibody pair was the crucial role affecting the sensitivity and specificity of the immunoassay.

The purified L-FABP analyte (100 μg/ml) was diluted to a range of concentrations of antibody pair with 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, and 6.4 μg/ml, the optimal antibody pair concentration was selected as 0.8 μg/ml according to the maximal signal-to-noise ratio, which not only could satisfy the clinical needs for detection but also could save the volume of L-FABP (Figure 3A). We also evaluated the incubation time of antibody pair, which was set as 15, 30, 45, and 60 min. The RLU was increased with increased incubation time for 45 min. After 45 min, the RLU leveled off with increasing incubation time. Therefore, 45 min was selected as the optimal reaction time for further experiment (Figure 3B).

3.3 | Evaluating analytical performance of L-FABP

3.3.1 | Construction of linear curve

L-FABP standards in the form of freeze-dried powder were dissolved with sterile PBS conducted by manufacturer protocol. The serially different concentrations of calibration samples were dissolved using human plasma matrix (2, 4, 8, 16, 32, 64, 128, 200, 256 ng/ml) and measured by chemiluminescent immunoassay. Under the optimal experimental conditions, standard curve was obtained (\( Y = 5418.6X^{-12193} \)) and the linear range was 2–160 ng/ml with an outstanding determination coefficient of 0.9971 (Figure 4A). The linear range could satisfy the clinical detection needs and provide helpful information without predilution of specimens.

3.3.2 | Determination of the minimum limit of detection

The minimum LOD is one of the most important characteristics to evaluate clinical practice of methodology. The calculated minimum LOD was determined by the results of 10 repeatedly blank assays with triple standard deviation. The minimum LOD could be expressed as LOD = 3Sa/b where Sa meant the standard deviation of the response values and b meant the slope of the standard curve.\(^{21}\) The limit of detection was 0.88 ng/ml.

3.3.3 | Evaluation of precision

The precision of this methodology was estimated based on repeated measurements of different concentrations of L-FABP standards solution, which was prepared by adding L-FABP standards into healthy human plasma. Three different concentrations of L-FABP standards (15 ng/ml, 50 ng/ml, and 100 ng/ml) were measured ten times a day for three days by chemiluminescent immunoassay. The results showed that the intra-analysis CVs of the three samples enrolled were 8.73%, 4.72%, and 3.79%, respectively, and the interanalysis CVs of the three samples enrolled were 13.47%, 7.28%, and 5.94%, respectively (Figure 4B). The repeatability results were highly acceptable and revealed the excellent analytical performance of this methodology.

3.3.4 | Evaluation of accuracy

In order to evaluate the accuracy of this methodology, recovery experiment was performed by adding different concentrations of L-FABP standards (15, 50, 100 ng/ml) into healthy human plasma and analyzed immediately. Simultaneously, five different concentrations (5, 20, 50, 100, 150 ng/ml) of L-FABP solution within the linear range were analyzed and the standard curve was obtained. The results showed that the recovery rates in three different concentration samples were
99.76%, 102.27%, and 96.92%, respectively, and suggested that the novel developed method for detecting L-FABP could be applied to clinical samples analysis without the interferences of other components.

3.3.5 | Matrix effect and interference experiment

To evaluate the influence of different matrixes to reactive system, the L-FABP standards (50 ng/ml) were added into samples containing three matrixes of plasma, urine, and BSA solution, respectively. L-FABP in different matrix solution was measured for three times. The detective mean value in plasma samples was 47.36 ng/ml, the detective mean value in urine samples was 51.13 ng/ml, and the detective mean value in BSA solution was 49.95 ng/ml. The inter CV among three different matrix samples was 4.29%. The results showed that different matrixes almost had no influence in detecting L-FABP. To validate whether L-FABP had the analytical selectivity for clinical application, we evaluated the interference experiments between L-FABP and other biomarkers of AKI including blood urea nitrogen (BUN), neutrophil gelatinase-associated lipid carrier proteins (NGAL), and CysC. Different concentrations of BUN, NGAL, and CysC exhibited negligible chemiluminescent signals, which were much lower than the chemiluminescent signal of L-FABP.

3.3.6 | Determination of the reference interval

The reference interval of L-FABP based on healthy population was constructed by chemiluminescent immunoassay. In order to explore the reference interval of L-FABP, 120 participants were enrolled in this study by detecting L-FABP of plasma samples, and calculating the 95% confidence interval to obtain the results as 0.88 ng/ml to 5.98 ng/ml.

3.3.7 | Detection of L-FABP in clinical samples

To explore the clinical application of L-FABP in critically ill patients, we analyzed L-FABP concentration between 15 AKI patients and 85 non-AKI patients and found that the median L-FABP concentration was significantly higher in AKI patients (median (IQR):10.65(6.74–14.56)) than non-AKI patients (median (IQR): 5.43(3.20–7.60)) (Figure 5A). When 5.95 ng/ml was selected as a cut-off value, the diagnostic sensitivity and specificity were 71.8% and 92.5%, respectively (Figure 5B).
Performance of L-FABP as a predictive and diagnostic biomarker for AKI

The median concentration of L-FABP was higher in patients with AKI (median (IQR): 6.15 ng/ml (4.88–7.15)) than in patients with non-AKI (median (IQR): 4.80 ng/ml (3.65–6.05)) on 3 days before AKI (Figure 6A). The median concentration of L-FABP was remarkably higher in patients with AKI (median (IQR): 6.98 ng/ml (5.26–7.95)) than in patients with non-AKI (median (IQR): 4.92 ng/ml (3.72–6.30)) on 2 days before AKI (Figure 6B). The median concentration of L-FABP was significantly higher in patients with AKI (median (IQR): 7.50 ng/ml (5.96–8.95)) than in patients with non-AKI (median (IQR): 5.30 ng/ml (4.72–6.30)) on 1 day before AKI (Figure 6C). To compare the difference between AKI cohort and non-AKI cohort by assessing
dynamic changes of L-FABP before AKI occurrence and the results showed that L-FABP was a potential predictor of AKI. The ROC curve analysis was used for assessing the predictive power of L-FABP for AKI. The area under ROC curve of L-FABP on 3 days before AKI was 0.6886, 95% CI: [0.5522, 0.8249], \( p = 0.0715 \) (Figure 6D). The area under ROC curve of L-FABP on 2 days before AKI was 0.7096, 95% CI: [0.5886, 0.8307], \( p = 0.0135 \) (Figure 6E). The area under ROC curve of L-FABP on 1 day before AKI was 0.7534, 95% CI: [0.7279, 0.8789], \( p < 0.0001 \) (Figure 6F). The results revealed that L-FABP was a potentially predictive biomarker of AKI.

The median concentration of L-FABP in patients with non-AKI (median (IQR): 4.70 ng/ml (3.79–5.10)) was remarkably lower than in those with AKI stage I (median (IQR): 10.20 ng/ml (8.90–12.65)), AKI stage II (median (IQR): 16.80 ng/ml (14.60–17.90)), and AKI stage III (median (IQR): 19.50 ng/ml (17.60–20.30)) (Figure 6G). Increasing concentration of L-FABP revealed the development of AKI and L-FABP was an excellent biomarker for diagnosing and indicating the severe degree of AKI.

### 3.5 Risk assessment performance of L-FABP before AKI occurrence

We used Restricted Cubic Spline plots to evaluate the association between the dynamic changes of L-FABP concentration and risk.
assessment of AKI occurrence. The higher L-FABP concentration was significantly associated with increased risk of AKI, \( p < 0.05 \) (Figure 6H). When L-FABP concentration was more than 5.7 ng/ml, the hazard ratio of AKI was more than 1, which was needed to attach great importance clinically. L-FABP was an excellent predictor for risk analysis of AKI. L-FABP levels were associated with the risk of AKI. Higher quartiles of L-FABP levels were independently associated with increasing risk of AKI. The highest quartile of L-FABP on 1 day before AKI was associated with increased odds for AKI by 92-fold compared with the lowest quartile. The highest quartile of L-FABP on 2 days before AKI was associated with increased odds for AKI by 65-fold compared with the lowest quartile. The highest quartile of L-FABP on 3 days before AKI was associated with increased odds for AKI by 56-fold compared with the lowest quartile; the results were shown in Table 2. When L-FABP levels were analyzed as a continuous variable, higher L-FABP was also associated with the development of severe AKI. The L-FABP level of stage II was associated with increased odds for AKI by 12-fold compared with stage I. The L-FABP level of stage III was associated with increased odds for AKI by 14-fold compared with stage I; the results were shown in Table 3.

### 4 | DISCUSSION

Chemiluminescent immunoassay was a newly developed immunological-based detection technique, which combined the advantages of enzyme immunoassay and radioimmunoassay.\(^22\) The outstanding advantages of double antibody sandwich based chemiluminescent immunoassay high sensitivity and specificity, excellent analytical selectivity, good repeatability and accuracy were being increasingly applied to material analysis.\(^24\) In this study, we constructed a rapid, sensitive, and practical chemiluminescent immunoassay detection method, which exhibited highly sensitive analytical performance, with a lower detection limit of 0.88 ng/ml compared with ELISA, where the limit of detection was 2.4 ng/ml.\(^27\) Highly automated and high-throughput analytical assay based on our chemiluminescent immunoassay replaced complicated operating procedures, giving a fast and convenient application in clinical practice and were suitable for large-scale sample measurements. Remarkably, biotin can bind avidin with high specificity, and biotin-avidin system has been widely applied in biomedical fields. Biotin-streptavidin system (modified biotin-avidin system) was used in our novel developed detection method to improve sensitivity by multistage amplifying function and to achieve the purpose of detecting trace antigen by streptavidin-enzyme color reaction.

Recently, increasing attention had been placed on AKI. Many diseases could cause AKI such as sepsis, multiple injury, and hepatic and renal insufficiency.\(^28\) AKI could easily progress to CKD without any intervention.\(^29\) Therefore, predicting and diagnosing AKI occurring and persistence was extremely important for preventing diseases. Based on the Chemiluminescent Immunoassay platform, we constructed the novel methodology to measure L-FABP with large-scale samples and resolved the problem which insufficient diagnostic performance of serum creatinine and urine output. However, there were deficiencies in the study which the sample size was not enough and the positive samples of AKI need to be increased. In addition, urine L-FABP may be a potential biomarker for prediction of AKI that was what we would do in the future. Nowadays, single biomarker had the disadvantages of insufficient prediction efficiency. The combination of multiple clinical indicators was able to improve clinically predictive efficiency of AKI. To construct a newly useful detection method and explore the clinical application of biomarkers were the direction of our future efforts.

### 5 | CONCLUSIONS

We developed a new, rapid, sensitive double-antibody sandwich-based chemiluminescent immunoassay to identify the plasma concentration of L-FABP. An automated, high-throughput detection method replaced the conventional manual detective method with simple and convenient experimental procedures. A wide linear range and high sensitivity, repeatability, and accuracy were the advantages of the proposed method. Furthermore, we conducted a preliminary

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**TABLE 2** Risk assessment of L-FABP for AKI by Logistic regression analysis

|                | 1 day before AKI (\(N = 525; 131-132\) per quartile) | 2 days before AKI (\(N = 437; 109-110\) per quartile) | 3 days before AKI (\(N = 348; 87-88\) per quartile) |
|----------------|------------------------------------------------------|------------------------------------------------------|------------------------------------------------------|
| Quartile 1     | \(1.0\) (reference)                                  | \(1.0\) (reference)                                   | \(1.0\) (reference)                                   |
| Quartile 2     | 54.87 [41.90, 67.83]                                  | 37.21 [29.29, 45.14]                                  | 37.69 [28.64, 46.74]                                  |
| Quartile 3     | 71.47 [54.51, 88.43]                                  | 48.34 [37.96, 58.71]                                  | 48.77 [36.95, 60.59]                                  |
| Quartile 4     | 92.71 [70.72, 114.70]                                 | 65.27 [51.28, 79.27]                                  | 65.90 [49.93, 81.87]                                  |

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**TABLE 3** Risk assessment of L-FABP for stages of AKI by Logistic regression analysis

|                | OR 95% CI       | \(p\)     |
|----------------|-----------------|----------|
| AKI group (\(N = 15; AKI stage 1: 10; AKI stage 2: 3; AKI stage 3: 2\)) |                 |          |
| AKI stage 1    | 1.0 (reference) |          |
| AKI stage 2    | 12.50 [1.22, 23.78] | 0.030   |
| AKI stage 3    | 14.73 [2.49, 26.97] | 0.018   |
study to explore the predictive and diagnostic value of L-FABP for AKI. The technique based on our new detection method provided remarkable advantages for detecting L-FABP continuously. These benefits highlight the application of L-FABP as a predictive and diagnostic biomarker for AKI. Looking toward future research, including multicenter and large-scale clinical trials, is urgently needed to validate the clinical application of L-FABP.

CONSENT FOR PUBLICATION
All authors have agreed to the consent of the manuscript.

DATA AVAILABILITY STATEMENT
The datasets used and/or analyzed during this study are available from the corresponding author on reasonable request.

CONFLICT OF INTERESTS
The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS
Tao Sun finished the manuscript and abstract; Shufang Qu, Tiancha Huang, Ying Ping, and Qinyan Lin collected clinical samples; Ying Cao and Weiwel Liu completed the figures and tables; Danhua Wang and Piaoping Kong consulted relevant literature and completed English revision; Zhihua Tao provided constructive feedback and guidance. All authors have read and approved the final manuscript.

ETHICS APPROVAL
All patients have written informed contents, and this study was approved by the Second Affiliated Hospital of Zhejiang University School of Medicine.

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