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**RESEARCH ARTICLE**

# Urinary neutrophil gelatinase-associated lipocalin and L-type fatty acid binding protein as diagnostic markers of early acute kidney injury after liver transplantation

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**Abstract**

*Objective*: We examined the value of two potential novel urinary biomarkers, neutrophil gelatinase-associated lipocalin (NGAL) and L-type fatty acid binding protein (L-FABP), in diagnosing acute kidney injury (AKI) in liver transplant recipients.

*Methods*: NGAL and L-FABP in urinary sample from Twenty-five patients before surgery and at 2, 4, 6, 12, 24, 48, 72 and 120 h after the anhepatic phase were tested. Standard statistics were used along with receiver-operating characteristic (ROC) analysis to evaluate the diagnostic value of selected markers.

*Results*: Urinary NGAL was only slightly elevated at 2 h in the non-AKI group while rose and stayed high from 2–6 h in the AKI group. However, urinary L-FABP rose transiently in both groups 2–120 h following surgery. The level of urinary NGAL presented differences at 2–6 h ($p < 0.05$) and urinary L-FABP at 4 h ($p < 0.05$) between AKI and non-AKI groups. ROC analysis showed that area under the curves (AUCs) of NGAL were 0.766, 0.773, and 0.773 at 2, 4 and 6 h respectively while 0.760 of L-FABP at 4 h.

*Conclusion*: Urinary NGAL rather than L-FABP appeared to be a sensitive and specific marker of AKI in liver transplant recipients.

**Keywords:** Growth factors/cytokines/inflammatory mediators, oxidative stress, renal disease

## Introduction

The development of liver transplantation has progressed rapidly during the last years, and vastly improved the outcomes of patients with end-stage liver disease (ESLD). However, perioperative complications remain a large risk for liver transplant recipients. Acute kidney injury (AKI), is a common complication and is known to increase both short and long term complications, including the risk of end-stage renal disease (ESRD) requiring dialysis, other comorbidity and mortality (Menon et al. 2004; Barri et al. 2009).

While current data suggest that early AKI is treatable if the diagnosis is made early (Schrier et al. 2004), a well established, validated and reliable marker of kidney injury in an early phase is lacking. ESLD and liver surgery furthermore reduce the usefulness of traditional biomarkers of AKI such as serum creatinine (Scr), blood urea nitrogen (BUN) and estimated glomerular filtration rate (GFR) (Orlando et al. 2002). Some complications such as muscle wasting and ascites could have some effect on the level of these biomarkers. Reductions in muscle mass and the ability to convert creatine to creatinine decrease creatinine concentrations in plasma. Ascites further contributes to overestimated GFR values by the Cockcroft–Gault formula because it increases body

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weight (but not muscle mass), which is included in the numerator of the formula. Recent studies have proposed neutrophil gelatinase-associated lipocalin (NGAL) and L-type fatty acid binding protein (L-FABP) as better diagnostic markers of AKI in nontransplant populations (Mishra et al. 2005; Wagener et al. 2006; Zappitelli et al. 2007; Portilla et al. 2008). In an attempt to establish the usefulness of these markers in the transplant setting, we examined patients undergoing liver transplant surgery in a prospective manner, collecting blood and urine during the first 5 days, with patient follow-up for 2 weeks.

## Patients and methods

### Patient population

After the study was approved by the Ethics Committee at the Shanghai Jiaotong University, informed consent was obtained from all patients participating in this study. We prospectively enrolled patients with ESLD who were scheduled to undergo primary liver transplantation including both deceased ($n = 11$) and living ($n = 14$) donor liver transplantation at the Shanghai Jiaotong University during the period from December 2007 to December 2008. Patients were excluded if (i) they were less than 18 years old or (ii) required a combined liver–kidney transplant or (iii) cardiac arrest, or death, occurred during surgery or within 48 h after the surgery or (iv) signs of chronic kidney disease (CKD) were present or AKI occurred before the surgery. According to the AKI Network (AKIN) criteria of AKI (Mehta et al. 2007), AKI was defined as an absolute increase in Scr of either 26.4 μmol/L or a percentage increase of 50% (1.5-fold from baseline) within 48 h after the operation. AKI occurred during 7 days after the operation was defined as postoperative AKI. Spot urine and blood samples were drawn from patients for the determination of Scr, urinary creatinine (Ucr), urine NGAL and urine L-FABP at the following nine time points: before operation 0 h, 2 h, 4 h, 6 h, 12 h, 24 h, 48 h, 72 h and 120 h after the anhepatic phase (release of post vena cava clamp). Urine and blood samples were centrifuged at 2000*g* for 5 min, and the supernatants stored in aliquots at –80°C. Serum and urine creatinine was measured by the hospital clinical laboratory.

### Clinical assessment of patients

Clinical practice was not changed during the study and all patients were treated according to established protocols. In all patients, immunosuppression was administrated according to an established protocol with prednisone, a calcineurin inhibitor (CNI) and mycophenolate mofetil (MMF). Steroid induction of 1.5 mg/kg was gradually tapered to 5 mg/d and tacrolimus was started a few hours after surgery, targeting a through level of tacrolimus of 8–12 ng/mL by the end of the first week post-transplant. MMF was started at 0.5–0.75 g twice per day.

Baseline demographic and clinical parameters included gender, age, diabetes mellitus, serum bilirubin, albumin and Scr. We also recorded intraoperative parameters, including operation times, volume of colloid infusion as well as of red blood cells. Postoperative data included duration of mechanical ventilation, and presence of infection and Systemic Inflammatory Response Syndrome (SIRS).

The clinical status of the patients was assessed regarding the liver status before surgery by using the model for ESLD (MELD) (Kamath et al. 2001), critical care status after surgery by using the Acute Physiology and Chronic Health Evaluation II (APACHE II).

### ELISA for quantitation of urinary NGAL

The levels of urinary NGAL were measured using the h-NGAL ELISA kit (R&D Systems, Inc. Minneapolis, MN). The NGAL protein standard or 50 μL of urine samples diluted up to 10-fold with the manufacturer-provided diluent in advance were transferred into a 96-well plate coated with a monoclonal antibody against h-NGAL. According to the manufacturer's instructions, the assay diluents and the conjugate reagent were added into every well step by step. After incubation, a substrate solution for the immunoperoxidase reaction was added to develop a color based on the amount of h-NGAL antigen present in the samples. The reaction was stopped using a stop solution. Urinary NGAL concentration was quantitated by measuring the absorbance of each well at 450 nm. Urinary NGAL level was expressed as the ratio of the urinary NGAL in ng/dL to urinary creatinine in mg/dL (ng/mg Ucr) to adjust for changes in urinary concentration.

### ELISA for quantitation of urinary L-FABP

The levels of urinary h-L-FABP were measured using the h-L-FABP ELISA kit (HyCult Biotechnology BV, Uden, the Netherlands). Urine samples were diluted up to 40-fold with diluent at first, and then were transferred to the 96-well plate. The measure procedure was similar with which of NGAL, and the urinary L-FABP level was also expressed as the ratio of the urinary L-FABP in ng/dL to urinary creatinine in mg/dL (ng/mgUcr).

### Statistical analysis

Kolmogorov–Smirnov test was used for estimating Gaussian distribution of the data and $p \geq 0.05$ was considered as normal distribution. Continuous variables were expressed as means and standard deviations or medians and interquartile range. Categorical variables were expressed as number (or as percentage, %). Differences between groups were analyzed with the independent-samples *t*-test or Mann–Whitney *U* test for continuous variables and with the $\chi^2$ test or Fisher's exact test for categorical variables. Differences between time points within the group were analyzed with paired-samples *t*-test or Wilcoxon rank sum test. The area under the curve (AUC) was calculated from a standard receiver-operating characteristic (ROC) plot. An AUC of 0.5 is no better than expected by chance, whereas a value of 1.0 signifies a perfect biomarker. A $p < 0.05$ was considered statistically significant. SPSS 15.0 (SPSS Inc., Chicago, IL, USA) was used to compute all statistical analyses and figures.

## Results

### Patients' characteristics

Figure 1 outlines the design of the study and patient flows. Briefly, we screened 33 patients, of which 25 were enrolled. Table 1 shows preoperative, perioperative and postoperative clinical characteristics in the patients retrospectively grouped according to whether they developed AKI or not. Briefly, there were no differences between the groups. The mean age of all patients was 47 ± 9 years, and the male/female ratio was 22/3. Eleven (44.0%) patients received deceased donor liver transplantation and fourteen (56.0%) received living donor liver transplantation. Twenty-three patients underwent transplantation due to complications related to hepatitis B virus infection (liver cirrhosis $n$ = 4; liver cirrhosis combined with early stage of hepatocellular carcinoma $n$ = 17; acute severe hepatitis $n$ = 1). The other two patients had primary biliary cirrhosis. All the patients had inferior vena cava clamped during anhepatic phase. No patient died and in no case renal replacement therapy (RRT) was required during the follow-up.

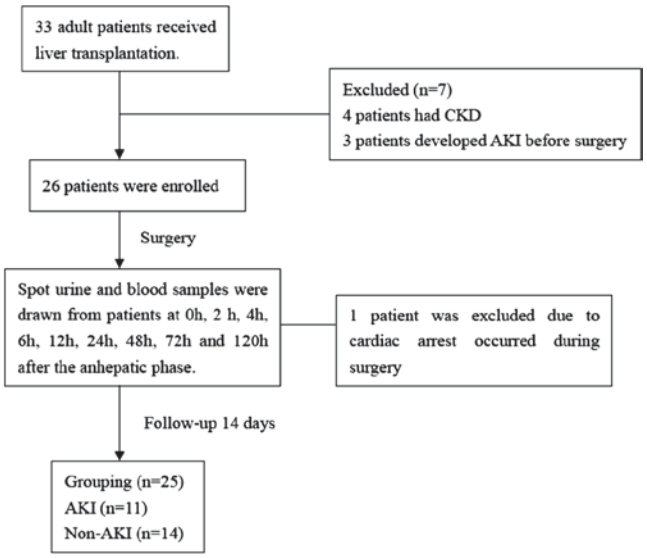


Figure 1. Study design.

### Postoperative renal marker changes

Following surgery, Scr rose slightly in non-AKI group 2–12 h after surgery compared to preoperation baseline, but this was higher and lasted longer in AKI patients (2–24 h). A significant difference was found at 24 h between two groups (Figure 2).

The time course of urinary NGAL and urinary L-FABP release after the anhepatic phase in 25 liver transplant recipients were analyzed according to whether they did or did not develop AKI. Urinary NGAL (Table 2, Figure 3) was only slightly elevated at 2 h in the non-AKI group, but rose and stayed high from 2–6 h in the AKI group. The differences between the two groups at 2 h, 4 h and 6 h were

Table 1. Preoperative, perioperative and postoperative clinical characteristics of liver transplant patients retrospectively grouped according to whether they developed AKI or not during 14 days after surgery.

| | Non-AKI ($n$ = 14) | AKI ($n$ = 11) | $p$ |
|---|---|---|---|
| Preoperative data | | | |
| Age (years) | 45.9 ± 8.05 | 48.5 ± 10.5 | NS |
| Gender (% males) | 11 (78.6%) | 11 (100%) | NS |
| Albumin (g/L) | 35.3 ± 6.8 | 35.4 ± 2.5 | NS |
| Scr (μmol/L) | 54.56 ± 11.61 | 49.76 ± 7.87 | NS |
| Serum bilirubin (mmol/L) | 25.30 (12.85–51.03) | 33.20 (30.50–81.70) | NS |
| MELD | 8.22 (3.94–10.72) | 8.26 (5.93–13.36) | NS |
| Diagnosis | | | NS |
| Hepatitis B | 13 (92.9%) | 10 (90.9%) | |
| Liver cirrhosis | 3 (21.4%) | 1 (9.1%) | |
| With HCC | 10 (71.4%) | 8 (72.7%) | |
| Acute severe hepatitis | 0 (0.0%) | 1 (9.1%) | |
| PBC | 1 (7.1%) | 1 (9.1%) | |
| Perioperative data | | | |
| Length of surgery (h) | 7.68 ± 1.16 | 7.49 ± 1.44 | NS |
| Anhepatic phase (min) | 91.50 (56.75–100.50) | 58.00 (45.00–121.00) | NS |
| Colloid infusion (L) | 2.66 ± 0.89 | 3.38 ± 1.61 | NS |
| RBC infusion (L) | 0.61 ± 0.64 | 0.89 ± 0.93 | NS |
| Postoperative data (until 14 days post-tx) | | | |
| 48 h APACHE II | 7.93 ± 2.34 | 8.27 ± 3.23 | NS |
| Mechanical ventilation time (h) | 6.17 (4.15–9.42) | 8.08 (4.58–10.50) | NS |
| Length of ICU stay (days) | 4.00 (3.00–4.50) | 5.00 (4.00–9.00) | NS |
| SIRS | 4 (28.6%) | 4 (36.4%) | NS |
| Infectious complication | 7 (50.0%) | 8 (72.7%) | NS |

AKI, acute kidney injury; Scr, serum creatinine; MELD, model for end-stage liver disease; PBC, primary biliary cirrhosis; HCC, hepatocellular carcinoma; RBC, red blood cells; APACHE II, Acute Physiology and Chronic Health Evaluation II; ICU, intensive care unit; SIRS, systemic inflammatory response syndrome.

significant ($p < 0.05$). Urinary L-FABP (Table 3, Figure 4) rose transiently in both groups 2–120 h following surgery. When comparing the values at each time points between the two groups, we found a significant difference at 4 h after anhepatic phase ($p < 0.05$).

### ROC analysis

Table 2 and Table 3 show the performance of the two biomarkers in diagnosing AKI at selected time points. Cut-off value of 43.02 ng/mgUcr at 2 h, 26.97 ng/mgUcr at 4 h and 17.19 ng/mgUcr at 6 h yielded good sensitivity and specificity of AKI diagnosis by urinary NGAL, as well as 3451.75 ng/mgUcr at 4 h by L-FABP. The AUC at different time points are shown in Table 4.

Table 2. Time course of urinary NGAL release after the anhepatic phase in 25 liver transplant recipients analyzed according to whether they did or did not develop AKI.

| Time points | Non-AKI ($n = 14$) NGAL ng/mgUcr[median (IQR)] | AKI ($n = 11$) NGAL ng/mg Ucr[median (IQR)] | $p$ (AKI vs non-AKI) |
|---|---|---|---|
| 0 h | 4.041 (0.79–12.58) | 5.13 (1.48–14.42) | NS |
| 2 h | 22.94 (8.69–46.23)* | 69.02 (29.79–237.29)* | 0.025 |
| 4 h | 12.66 (8.91–22.78) | 29.34 (16.06–536.91)* | 0.021 |
| 6 h | 11.84 (6.57–20.10) | 34.23 (11.47–81.26)* | 0.021 |
| 12 h | 10.49 (5.89–24.18) | 12.21 (10.73–66.02) | NS |
| 24 h | 16.15 (6.70–30.28) | 19.65 (7.78–58.75) | NS |
| 48 h | 14.14 (6.39–24.21) | 15.17 (5.56–25.74) | NS |
| 72 h | 13.56 (7.71–21.22) | 21.64 (5.49–52.32) | NS |
| 120 h | 20.57 (7.78–41.55) | 15.51 (14.41–44.29) | NS |

AKI, acute kidney injury; NGAL, neutrophil gelatinase-associated lipocalin; IQR, interquartile range.
*$p < 0.05$ compared to baseline (0 h).

## Discussion

In the study of the value of urinary NGAL and L-FABP as early diagnostic tools for AKI following liver transplantation, we report here that NGAL in the urine of patients that later develop AKI consistently but transiently elevates 2–12 h after reperfusion of the liver graft. The discriminatory power as indicated by the AUC was good at these time points.

As we know, human NGAL was originally identified as a 25 kDa protein that is involved in natural immunity (Kjeldsen et al. 1993; Fjaertoft et al. 2005). However, NGAL is known to be an acute-phase reactant released from multiple tissues (Cowland et al. 2003; Playford et al. 2006). In the kidney tubules, NGAL is upregulated within a few hours after harmful stimuli of various types (Mishra et al. 2003, 2004). This has led to a number of studies evaluating NGAL as a biomarker of AKI (Mishra et al. 2005; Bachorzewska-Gajewska et al. 2006; Wagener et al. 2006; Hirsch et al. 2007). In recent years, either serum or urinary NGAL was shown to be of benefit in patients with AKI after liver transplantation (Niemann et al. 2009; Portal et al. 2010; Wagener et al. 2011). The predictive power of urinary NGAL 3 h after liver transplantation described by Wagener et al. was similar to ours. However, in our study, urinary NGAL levels at 2 h after anhepatic phase increased not only in AKI group but also

Table 3. Time course of urinary L-FABP release after the anhepatic phase in 25 liver transplant recipients analyzed according to whether they did or did not develop AKI.

| Time points | Non-AKI ($n = 14$) L-FABP ng/mgUcr [median, (IQR)] | AKI ($n = 11$) L-FABP ng/mgUcr [median, (IQR)] | $p$ (AKI vs non-AKI) |
|---|---|---|---|
| 0 h | 2.03 (0.15–19.43) | 9.98 (0.05–53.04) | NS |
| 2 h | 3370.53 (1790.32–4248.85)* | 3243.84 (2718.99–4027.80)* | NS |
| 4 h | 2361.41 (1036.89–4048.93)* | 5246.97 (2406.33–7688.21)* | 0.029 |
| 6 h | 1170.17 (821.28–1601.97)* | 1695.95 (1272.11–4410.97)* | NS |
| 12 h | 698.50 (588.22–1057.71)* | 724.84 (525.64–1790.53)* | NS |
| 24 h | 532.29 (266.43–639.20)* | 383.99 (193.17–876.80)* | NS |
| 48 h | 258.12 (60.25–414.99)* | 175.52 (21.47–559.49)* | NS |
| 72 h | 154.33 (75.77–437.40)* | 210.21 (32.30–595.27)* | NS |
| 120 h | 239.15 (138.05–768.43)* | 245.44 (40.59–325.08)* | NS |

AKI, acute kidney injury; IQR, interquartile range; L-FABP, L-type fatty acid binding protein.
*$p < 0.05$ compared to baseline (0 h).

Table 4. Output from ROC curve analysis, demonstrating sensitivity and specificity of urinary NGAL and L-FABP as predictors – at different time points – of which patients that did or did not develop AKI.

| Time points | Cut-off value (ng/mgUcr) | Sensitivity | Specificity | AUC | $p$ | 95% CI |
|---|---|---|---|---|---|---|
| NGAL | | | | | | |
| 2 h | 43.02 | 0.727 | 0.786 | 0.766 | 0.025 | 0.577–.955 |
| 4 h | 26.97 | 0.636 | 0.857 | 0.773 | 0.021 | 0.588–0.957 |
| 6 h | 17.19 | 0.727 | 0.714 | 0.773 | 0.021 | 0.580–0.965 |
| L-FABP | | | | | | |
| 4 h | 3451.75 | 0.727 | 0.714 | 0.760 | 0.029 | 0.554–0.965 |

AUC, area under the curve; L-FABP, L-type fatty acid binding protein; NGAL, neutrophil gelatinase-associated lipocalin.

in non-AKI group. This may be due to the technique we used in the surgery. Complete occlusion of the inferior vena cava can result in a renal outflow obstruction, and experimentally, renal vein outflow obstruction has been shown to cause severe renal injury despite preserved arterial renal inflow several (Park et al. 2008). A maintenance of partial flow, by such as piggyback technique can protect the kidneys during the anhepatic phase (Wagener et al. 2011). Signs of subclinical kidney tubule injury could reflect hyperexpression of NGAL protein in the kidney tubules within a short time rather than a decline of kidney function leading to increase of serum creatinine (Mishra et al. 2003). Why the upregulation of NGAL expression occurs in renal ischemia is not clear but it could perhaps represent a defense mechanism in response to acute renal necrosis (ATN) (Mori et al. 2005). This is a further evidence that serum creatinine is much less sensitive to detect renal injury than urinary NGAL. In our study, NGAL compared favorably to L-FABP, rising earlier than the latter and lasting until 12 h after surgery, which likely more predictable to AKI. The ROC values are impressive, but need to be validated in larger studies.

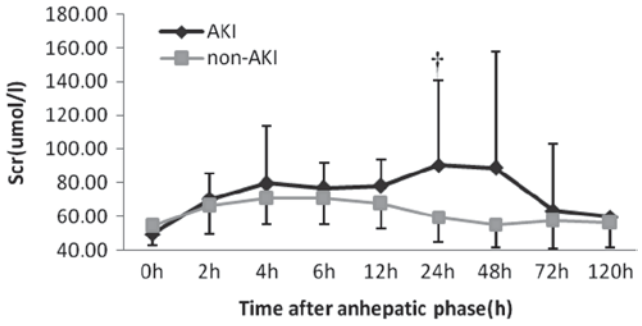


Figure 2. Postoperative changes in serum creatinine (Scr) following the anhepatic phase in liver transplant recipients grouped by whether they did ($n = 11$) or did not ($n = 14$) develop AKI. Whiskers show standard deviations, SD. †Significant difference ($p < 0.05$).

Fatty acid binding proteins (FABPs) are small (15 kDa) cytoplasmic proteins abundantly expressed in all tissues with active fatty acid metabolism (Glatz & van der Vusse 1996). Liver-type FABP (L-FABP) as the other biomarker investigated in our study is one type of the proteins identified in the proximal tubule of human kidney, and has been found to be a potential biomarker in a number of pathologic conditions, including CKD, diabetic nephropathy, IgA nephropathy, obstructive nephropathy and contrast nephropathy (Zager et al. 2005; Nakamura et al. 2005a, 2005b, 2006a, 2006b; Kamijo-Ikemori et al. 2006; Kamijo et al. 2006). Using human-L-FABP

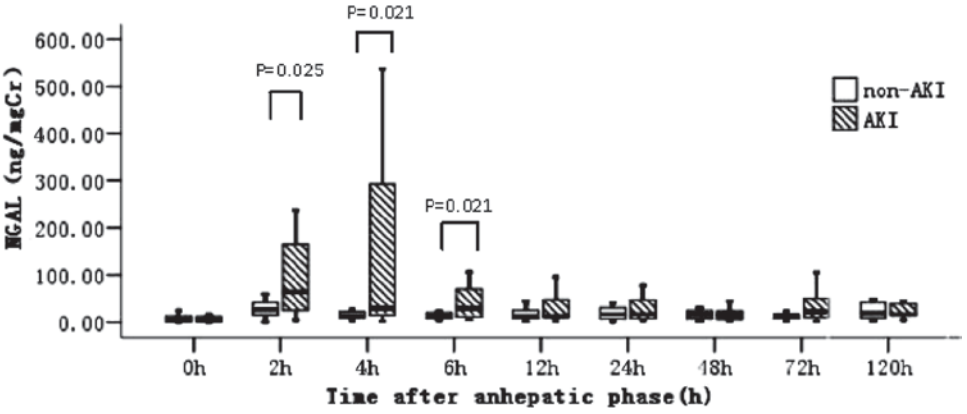


Figure 3. Postoperative changes in urinary neutrophil gelatinase-associated lipocalin (NGAL) following the anhepatic phase in liver transplant recipients grouped by whether they did ($n = 11$) or did not ($n = 14$) develop AKI. Whiskers show interquartile range (IQR).

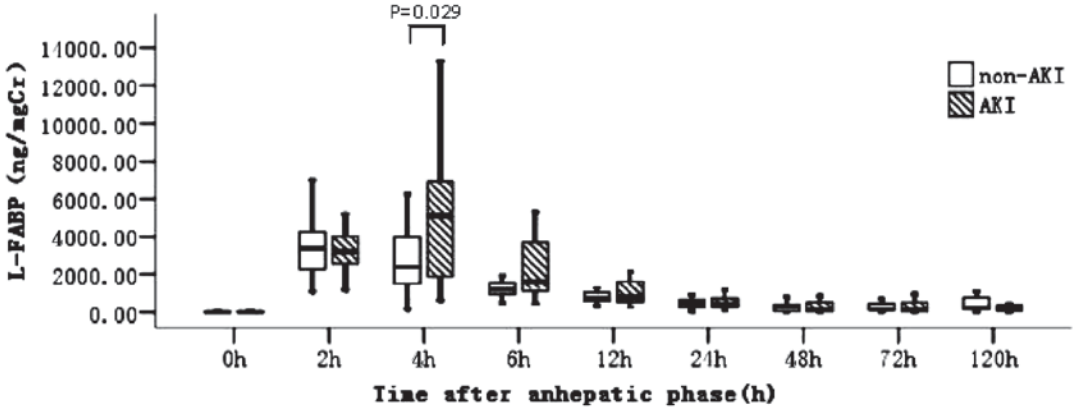


Figure 4. Postoperative changes in L-type fatty acid binding protein (L-FABP) following the anhepatic phase in liver transplant recipients grouped by whether they did ($n = 11$) or did not ($n = 14$) develop acute kidney injury (AKI). Whiskers show IQR.

transgenic mice and the model of ischemia reperfusion injury, Yamamoto et al. (2007) showed that an increase in urinary L-FABP after ischemic-reperfusion injury may be used as a biomarker of acute ischemic injury. This finding was confirmed in a pediatric cardiac surgery setting (Portilla et al. 2008). An increase of L-FABP levels of about 94- and 45-fold at 4 and 12 h, respectively following surgery were noted in the patients who developed AKI, and urinary L-FABP levels at 4 h after surgery was found to be an independent risk indicator with an AUC of 81% in ROC analysis. In the present study, urinary L-FABP, to the best of our knowledge, was the first to be assessed as diagnostic markers of AKI after liver transplantation. We found the changes in urinary L-FABP compared to preoperative levels were large both in patients with and without AKI. And differences between groups were significant at only one time point (4 h).

It may be difficult to interpret absolute levels indicative of AKI.The rapidly elevated urinary L-FABP after liver reperfusion maybe partly associated with increased filtration of high serum L-FABP levels. Besides in the kidney, L-FABP is mainly found in hepatocytes. In physiological condition, liver L-FABP is present in the circulation, filtrated from glomerulus and reabsorbed in proximal tubule mediated by megalin, a multiligand endocytic receptor (Saito et al. 1994). Although Portilla et al. (2008) has demonstrated that increased urinary L-FABP levels after cardiac surgery in AKI patients represent an increased in the shedding of proximal tubule L-FABP rather than just reflecting increased filtration of high serum levels, there was no report about interpretation of urinary L-FABP in liver transplant patients. Patients with liver damage have an elevated serum L-FABP level (Saito et al. 1994; Pelsers et al. 2002; Mori et al. 2005) and in a pig model of liver transplantation, an elevated serum L-FABP level was observed early after reperfusion (Monbaliu et al. 2005). We assumed that the elevation of serum L-FABP may result in an increased amount of glomerular filtration whereas the endocytosis of megalin is attenuated during the process of renal ischemia reperfusion (Negishi et al. 2007), which might influence the predictive ability of urinary L-FABP in AKI. Further studies to elucidate the origin of urinary L-FABP after liver transplantation were needed.

Several limitations should be acknowledged. First, our data is derived from a relatively small number of patients from a single center. Second, the prevalence of severe AKI was low and no patient needed RRT. Finally, although data was prospectively collected, it was analyzed retrospectively and thus was not used to influence therapy. Future interventional studies will have to elucidate if aggressively treating patients with a high NGAL can reduce the incidence and consequences of AKI in liver transplant recipients.

In summary, we report that urinary NGAL rather than L-FABP appeared to be a sensitive and specific marker of AKI in liver transplant recipients; however, these data need to be validated in larger prospective studies.



## Declaration of interest

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