

An AKI biomarker lipocalin 2 in the blood derives from the kidney in renal injury but from neutrophils in normal and infected conditions

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Abstract*Background:*

Lipocalin 2 (LCN2, neutrophil gelatinase-associated lipocalin or NGAL) is a secretory protein discovered from neutrophils, which accumulates in the blood and urine during acute kidney injury (AKI) and in the blood by bacterial infection. Little is known about the tissue source and molecular forms of this protein under normal and pathophysiologic conditions.

Methods:

By sandwich ELISA, serum and urinary LCN2 levels were measured in 36 patients with hematologic malignancies who transiently became neutropenic by stem cell transplantation (SCT). To evaluate contribution of neutrophil-derived LCN2 in the physiologic blood LCN2 concentrations, we examined CCAAT/enhancer binding protein ϵ (C/EBP ϵ) knockout mice, which lack mature neutrophils.

Results:

In patients without AKI and bacterial infection, at 1 week after SCT, the median blood neutrophil counts became zero and serum LCN2 levels were decreased by $76 \pm 6\%$ ($p < 0.01$), but urinary LCN2 levels were not altered. During neutropenic conditions, bacterial infection caused only a modest rise of serum LCN2 but AKI produced a marked rise of serum and urinary LCN2 levels. Serum LCN2 concentrations in C/EBP ϵ knockout mice were reduced by $66 \pm 11\%$ compared to wild-type mice ($p < 0.05$). Blood LCN2 existed predominantly in high-molecular-weight forms (> 100 kDa), while urinary LCN2 was mainly in low-molecular-weight forms.

Conclusion:

Our findings suggest that neutrophils are the major source of circulating LCN2 in normal and infected conditions, whereas blood and urinary LCN2 mainly derives from the kidney during AKI, and that the molecular forms and regulation of blood and urinary LCN2 are clearly distinct.

Introduction

Lipocalin 2 (LCN2 or neutrophil gelatinase-associated lipocalin) was originally purified from activated neutrophils [1, 2]. LCN2 gene expression is detected not only in neutrophils, but also in various normal tissues, such as lung, liver, and adipose tissue [2-4]. Its expression is markedly upregulated by renal injury [5-8] and bacterial infection [9]. LCN2 is now known to exert a broad spectrum of biological activities including host defense [9], kidney differentiation [10] and modulation of organ damage [5].

Blood and urinary levels of LCN2 have been extensively studied as very promising biomarkers for an early diagnosis of acute kidney injury (AKI) [2, 6, 7] and for monitoring of chronic kidney disease severity [11, 12], which may revolutionize our clinical practice in the near future. Bacterial infection also causes mild elevation of blood LCN2 levels [13, 14]. Thus, neutrophils and injured kidneys are two major candidate sites of LCN2 release in diseased conditions. Therefore, to make clinical judgment based upon LCN2 levels in the blood or urine, it is important to understand tissue source of LCN2. Furthermore, a fraction of neutrophil-derived LCN2 is covalently bound to gelatinase B (or metalloproteinase-9) [15, 16], but the details about the molecular forms of LCN2 in body fluid largely remain unknown.

In the present study, we examined whether neutrophils contribute to blood LCN2 levels in AKI or in bacterial infection by analyzing a unique subset of patients who were neutropenic after stem cell transplantation (SCT) [17]. Since not all cases were in complete remission status before SCT, we also studied wild-type and CCAAT/enhancer binding protein ϵ (C/EBP ϵ) knockout mice [18], which reflect normal and neutropenic conditions, respectively, in the absence of hematologic malignancies. To study the mode of existence of LCN2, we separated serum and urine with 100-kDa cutoff ultrafiltration membranes and measured the levels of high- and low-molecular-weight (HMW and LMW) LCN2 forms. Furthermore, we examined LCN2/gelatinase B complex.

Methods

Patients

Patients with hematologic malignancies undergoing autologous or allogeneic SCTs at Kyoto University Hospital (Electronic Supplementary Material Table S1), healthy subjects and patients with renal disorders were enrolled under written informed consent. This prospective, observational study was approved by the ethical committee on human research of Kyoto University Graduate School of Medicine (approval number E-541). AKI was defined by $\geq 50\%$ elevation of serum creatinine level during the observation period in comparison with the level before SCT. Bacterial infection was determined by development of pyrexia ($> 38^{\circ}\text{C}$) together with either a positive blood culture test or clinical symptoms highly suggestive of local infection or septic shock.

Animals

All animal experiments and protocols were approved by our institutional animal care and use committee. Sera were collected from C/EBP $\epsilon^{-/-}$, C/EBP $\epsilon^{+/+}$ mice and their wild-type littermates in a mixed background of 129SV x NIH Black Swiss at 8 to 10 weeks of age [18].

Expression vectors and promoter-reporter gene constructs

The human CCAAT/enhancer binding protein (C/EBP) α cDNA [19] and the human C/EBP ϵ 32 cDNA [20] were sub-cloned into pCDNA3.1+ (Invitrogen, Carlsbad, CA). A luciferase reporter construct was prepared in pGL3 basic vector (Promega, Madison, WI), containing sequences between nucleotide positions -900 to +51 of human LCN2 promoter region [4].

Reporter assay

In 12-well plates, 1×10^5 293T cells were seeded per well one day before transfection. Either C/EBP α , C/EBP ϵ 32 or mock vector (pcDNA3.1+) was transfected into 293T cells with the reporter vector using TransFectin Lipid Reagent (BioRad, Hercules, CA). Forty-eight hours later, cell lysate was collected and measured for luciferase activity using Dual Luciferase Assay System (Promega, Madison, WI).

ELISA

Human and murine LCN2 concentrations in the serum or urine were determined once a week by sandwich ELISA (kits 036 and 042, BioPorto, Gentofte, Denmark). To separate serum and urine by molecular weights, samples were passed through 100-kDa cutoff filter (Amicon YM-100; Millipore Corp., Billerica, MA). LCN2/gelatinase B complex levels from humans were measured by ELISA (Quantikine, R&D Systems, Minneapolis, MN).

Western blot analysis

YM-100 flow-through of mouse serum was separated by SDS-polyacrylamide gel electrophoresis, transferred onto polyvinylidene difluoride membranes, incubated with goat anti-mouse Lcn2 antibody (R&D Systems, Minneapolis, MN), with peroxidase-conjugated anti-goat antibody, and detection was carried out using chemiluminescence. As standards, recombinant mouse Lcn2 protein synthesized in BL21 bacteria was loaded [5].

Statistical analysis

Values are expressed as means \pm SEM, or median (interquartile range) when appropriate. Differences between repeated measures were assessed by one-way ANOVA with Bonferroni's post test. Comparison between two groups was carried out by unpaired Student's t test. The correlation between blood neutrophil counts and LCN2 levels was tested by Pearson's correlation coefficient. Cross-sectional time-series regression model was used in univariate and multivariate analyses to evaluate potential factors which were associated with the level of LCN2. Confounders that were analyzed included the number of neutrophils, lymphocytes and platelets, the levels of hemoglobin, serum creatinine, C-reactive protein, and body mass index (defined as body weight divided by square of height), and weeks after SCT. Significant independent variables in univariate analysis, as well as weeks, were included in multivariate analysis. Standardized coefficients were calculated to evaluate which of the independent variables have greater effects on the dependent variable. p values of less than 0.05 were considered statistically significant. All statistical analyses were performed using Stata software version 11 (Stata Corp., College Station, Texas, USA).

Results

Blood and urine human LCN2 levels in patients undergoing stem cell transplantation

We studied 36 patients who underwent SCT for their hematological malignancies. The time course of serum and urinary LCN2 levels during periods from pre-transplantation (between -2 to -1 weeks) to 4 weeks after autologous or allogeneic SCT were examined (Fig. S1). These patients were categorized into 4 groups based upon the presence or absence of AKI or bacterial infection (Table 1). Twelve patients (33%) were categorized as Group 1 [bacterial infection (-), AKI (-)]. At 1 week, median blood neutrophil count became $0/\mu\text{l}$, and serum LCN2 level was reduced by $76 \pm 6\%$ (from 63 ± 15 to 10 ± 1 ng/ml, $p < 0.01$, Fig. 1). Among the 4 groups, the general trend of neutrophil counts and serum LCN2 concentrations during the observation period was similar, with the lowest levels at 1 and 2 weeks and gradual recovery towards 4 weeks (Figs. 1, 2). These findings suggest that the predominant source of circulating LCN2 is blood neutrophils but substantial amount of a non-neutrophil pool also exists.

Twelve subjects (33%) were categorized as Group 2 [bacterial infection (+), AKI (-)] (Fig. 1). Bacterial infection was diagnosed on day 8.2 ± 1.7 (Fig. S1). Peak serum C-reactive protein (CRP) levels were 8.8-fold higher in Group 2 as compared to Group 1 ($p < 0.05$, Table 1). The time course of blood neutrophil counts and serum LCN2 levels was roughly similar as compared to Group 1, but neutrophil counts were significantly higher at 3 weeks in Group 2 ($p < 0.05$, Fig. 1). In Group 2, 6 patients had zero neutrophil counts not only at 1 week but also at 2 weeks and developed bacterial infection on day 8.2 ± 1.4 . Their serum LCN2 levels at 1 and 2 weeks were 12 ± 4 and 25 ± 9 ng/ml, respectively. These findings suggest that bacterial infection caused small amount of LCN2 release from non-myeloid tissues, which could be from the lung, liver, spleen or adipose tissue [2-4]. The urinary LCN2 excretion in Groups 1 and 2, unlike serum LCN2 levels, did not decrease during the first week (which was neutrophil-depleted period), indicating that circulating and urinary LCN2 levels were regulated in distinct manners (Fig. 1).

Most patients with AKI also suffered from bacterial infection, and only 2 patients (6%) belonged to Group 3 [bacterial infection (-), AKI (+)](Fig. 2). These 2 patients developed AKI at 3 and 4 weeks, respectively, and their serum LCN2 levels at 4 weeks tended to be higher compared to patients without AKI (Groups 1 and 2), but the sample number in Group 3 was too small for statistical analysis.

Ten patients (28%) were classified into Group 4 [bacterial infection (+), AKI (+)]. The peak serum creatinine levels were 2.9-fold higher in Group 4 compared to Group 1 ($p < 0.05$, Table 1). Diagnoses of bacterial infection and AKI were made on days 6.9 ± 1.2 and 8.3 ± 1.4 , respectively. Blood neutrophil counts became almost zero at 1 and 2 weeks as similar to the cases in Groups 1-3. However, during this nadir period especially at 2 weeks, serum LCN2 levels became much higher than the levels before SCT (Fig. 2, Fig. S1). Three patients in Group 4 and one in Group 3 started to receive continuous hemodiafiltration (CHDF) at various time points due to oliguria or hyperkalemia (Fig. S2). The timing of CHDF initiation was closely associated with elevation in the serum and urinary LCN2 levels by log orders of magnitude, occasionally reaching the levels above 300 ng/ml or 2,000 $\mu\text{g/gCr}$, respectively. These cases clearly show that AKI causes a steep elevation in serum and urinary LCN2 levels, even in the absence of neutrophils.

Determinants of serum LCN2 levels

We identified a strong positive correlation between neutrophil counts and serum LCN2 levels among samples excluding those collected when AKI was present (Fig. 3). Blood samples during AKI contained larger amounts of

LCN2 than ones with no AKI. Multivariate analyses for all samples (with and without AKI) showed that the serum levels of LCN2 were significantly associated with neutrophil counts (standardized coefficient 0.57, $p < 0.001$) as well as with serum CRP levels (standardized coefficient 0.16, $p < 0.05$, Table 2). The significant positive correlation between serum LCN2 and creatinine levels in univariate analysis was lost in multivariate analysis, likely because samples with elevated serum creatinine levels were partially enriched in those with low blood neutrophil counts (Figs. S1, S2).

Characterization of molecular forms of blood and urine LCN2

LCN2 protein in serum or urine may exist in several molecular forms, including a 25-kDa monomer, a 46-kDa homodimer and a 135-kDa heterodimer with gelatinase B (or MMP-9) [1, 16]. Serum and urine were passed through 100-kDa cutoff membrane to separate LCN2 into HMW and LMW forms (Fig. S3). Approximately 82% of serum LCN2 existed as HMW form, and presence of neither bacterial infection nor AKI affected the ratios (Fig. 4, Fig. S3). On the other hand, approximately 99% of urinary LCN2 was in LMW form in most patients who underwent SCT (both before and after SCT) and in healthy subjects (data not shown). Exclusively, urine from SCT patients who developed AKI with overt proteinuria (urinary protein level $> 1\text{g/g}$ creatinine) contained as much as 37% of HMW form. Similarly, patients with nephrotic range proteinuria due to chronic kidney disease had large amount of HMW LCN2 in the urine (Fig. 4). These findings suggest that, if glomerular size barrier is functioning normally, only a small fraction of HMW LCN2 in the blood (as is the case with 60-kDa albumin) is filtered through glomeruli and trace amount, if any, is excreted into urine. Thus, circulating LCN2, due to its large molecular size, likely has a much longer blood half-life than that of LCN2 monomer (which is about 10 min) [21].

A portion of LCN2 secreted from neutrophils is covalently bound to gelatinase B [15, 16] and LCN2/gelatinase B complex is one of the candidate forms of HMW LCN2. We examined the content of this complex in the serum and urine (Table S2). Ratio of complex among total LCN2 immunoreactivity was less than 30% in serum of healthy subjects and patients undergoing SCT. Furthermore, the concentration of complex in urine was quite low ($< 2\text{ ng/ml}$, and typically $< 3\%$ of total urine LCN2 immunoreactivity). These findings suggest that the majority of HMW LCN2 in the blood exists in forms other than LCN2/gelatinase B complex. Furthermore, the ratio of complex in the blood was largely and temporarily reduced when patients were in neutropenic periods (at 1 week after SCT), suggesting that bone marrow or peripheral neutrophils are important sources of circulating LCN2/gelatinase B complex.

Serum Lcn2 levels in C/EBP ϵ knockout mice

As described above, serum LCN2 levels became $24 \pm 6\%$ of baseline levels during neutropenic conditions in patients who underwent SCT. To investigate impact of presence of neutrophils upon circulating LCN2 concentrations in normal conditions, we examined genetically mutant mice which lack mature neutrophils. C/EBP ϵ is a transcriptional factor which is crucial for neutrophil and eosinophil differentiation [18, 20, 22]. Given that LCN2 mRNA was abundantly expressed in human myeloid cell lines expressing C/EBP ϵ [2, 22], we first assessed the effects of C/EBP ϵ on promoter activity of human LCN2 gene. In luciferase assay, overexpression of C/EBP ϵ , as well as C/EBP α , significantly enhanced the promoter activity of LCN2 gene (Fig. 5a). Since C/EBP ϵ ^{-/-} mice have severely impaired terminal differentiation of neutrophils [18, 22], these mice were utilized to determine the impact

of C/EBP ϵ -dependent neutrophil maturation on steady-state serum Lcn2 levels. By ELISA, serum Lcn2 levels in C/EBP $\epsilon^{-/-}$ and C/EBP $\epsilon^{+/-}$ mice were reduced by $66 \pm 11\%$ ($p < 0.05$) and by $34 \pm 12\%$, respectively, as compared to C/EBP $\epsilon^{+/+}$ animals (Fig. 5b). Similar differences among genotypes were observed by Western blot of LMW (< 100 kDa) fraction of the serum (Fig. 5c). These findings indicate that C/EBP ϵ is essential for maintaining steady-state serum LCN2 levels in mice. As to molecular forms, approximately 72% of serum Lcn2 was in HMW form, and the ratio was not altered by absence of neutrophils in KO mice (Fig. 5d).

Discussion

Here, we have shown that serum LCN2 levels are decreased by 76% during neutropenic conditions after SCT, consistently with our present findings in C/EBP ϵ KO mice, which lack functionally mature neutrophils [18, 22]. Furthermore, serum LCN2 levels showed a strong correlation with blood neutrophil counts. These findings show, for the first time to our knowledge, that circulating neutrophils are the predominant source of steady-state blood LCN2.

LCN2 plays an essential role in host defense by inhibiting the growth of bacteria such as *Escherichia coli* and *Mycobacterium tuberculosis* [9, 23]. Therefore, not only neutropenia but also reduced circulating LCN2 levels may contribute to the high susceptibility of patients to infection during the neutrophil nadir periods after SCT. To date, it is not known whether subjects with supranormal circulating LCN2 levels are super-protected against infection.

When serum and urine LCN2 was separated into HMW and LMW forms, HMW LCN2 was the major form in blood, while urinary LCN2 consisted almost exclusively of LMW forms. These findings were quite surprising, since blood and urinary LCN2 levels are both elevated early in the course of AKI and both of these markers have been considered to be useful biomarkers of AKI [2, 6]. During nadir periods of the neutrophil counts, bacterial infection caused minimal elevation in serum LCN2 levels, but AKI lead to remarkable elevation in serum and urinary LCN2 levels. These findings suggest that the major source of serum LCN2 is the neutrophils in healthy and infected conditions, whereas the kidneys, especially the nephron segments of the thick ascending limbs of Henle and collecting ducts [2, 8, 11], are the main source of serum and urinary LCN2 in AKI. Cai et al. examined molecular forms of LCN2 in the urine and reported that renal tubules mainly secrete monomer LCN2, whereas neutrophils predominantly release dimer LCN2 [24]. Information concerning LCN2 in the blood was not provided in their work [24]. Of note, mature neutrophils contain large amount of LCN2 protein in the secretory granules but LCN2 mRNA expression is lost in these cells [1, 2, 25], making it very difficult to quantitatively evaluate neutrophil-derived LCN2 at the mRNA level. In patients with overt proteinuria (which suggests the presence of severe glomerular injury), HMW urinary LCN2 proteins were observed (Fig. 4b). At least some of HMW urinary LCN2 proteins are presumed to derive from the blood. Recently, we have been able to purify and identify several LCN2-binding proteins in the urine from patients with chronic kidney disease [26].

How is LCN2 synthesized in the kidney secreted in the blood or excreted in the urine deserves to be discussed. When we stained Lcn2 protein in injured mouse kidneys in a previous study, we found 2 staining patterns [11]. One was a granular pattern along apical side of proximal tubules, which appears to reflect blood-driven, reabsorbed Lcn2 protein. The other was diffusely distributed in the cytoplasm of distal nephron cells, and we speculate that Lcn2 protein in this compartment is released into the urine or circulation, at least partially, through a non-specific pathway, not depending upon secretory granules. Indeed, wild-type kidney transplanted into Lcn2 KO mice does release Lcn2 protein in the urine (potentially through circulation) after induction of ischemic kidney injury [8].

A portion (15%) of LCN2 in neutrophil secretory granules co-localizes with gelatinase B [3] and forms heterodimer with gelatinase B [1], preserving gelatinase B from degradation [27]. When we examined the concentration of LCN2/gelatinase B complex in the total LCN2 immunoreactivities in the blood of healthy subjects and patients undergoing SCT, the complex occupied less than 30%. We also found that the ratio of the complex is largely reduced during neutropenic periods. These findings suggest that neutrophils are an important source of

LCN2/gelatinase B complex in the blood but the complex only constitutes a small fraction of circulating LCN2.

There are several limitations in this study. The present study was mainly focused to longitudinal analysis of patients undergoing SCT, and the numbers of patients with hematologic or renal disorders and healthy subjects were small. Since severity of bacterial infection is generally larger when it is associated with AKI, higher peak serum and urinary LCN2 levels in Group 4 [bacterial infection (+), AKI (+)] compared to Group 2 [bacterial infection (+), AKI (-)] may have been caused not only by complicating AKI but also by more severe infection.

In conclusion, neutrophils are the predominant source of circulating LCN2 in physiological conditions, which may play an important role in the prevention of bacterial infection. In AKI, serum LCN2 proteins are dramatically increased even among patients in neutropenic states, suggesting that injured kidneys are major source of circulating LCN2 in pathologic conditions. The present study brings new insights into our understanding of the complicated regulation and clinical implication of blood or urinary LCN2 concentrations as biomarkers of AKI.

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Conflict of interest

K.M. and J.B. are a part of patent co-inventors for LCN2 as a diagnostic marker of renal failure. The other authors have no conflicts of interest to declare.

References

1. Kjeldsen L, Johnsen AH, Sengelov H, Borregaard N. Isolation and primary structure of NGAL, a novel protein associated with human neutrophil gelatinase. *J Biol Chem.* 1993,268:10425-32.
2. Mori K, Nakao K. Neutrophil gelatinase-associated lipocalin as the real-time indicator of active kidney damage. *Kidney Int.* 2007,71:967-70.
3. Cowland JB, Borregaard N. The individual regulation of granule protein mRNA levels during neutrophil maturation explains the heterogeneity of neutrophil granules. *J Leukoc Biol.* 1999,66:989-95.
4. Cowland JB, Borregaard N. Molecular characterization and pattern of tissue expression of the gene for neutrophil gelatinase-associated lipocalin from humans. *Genomics.* 1997,45:17-23.
5. Mori K, Lee HT, Rapoport D, Drexler IR, Foster K, Yang J, Schmidt-Ott KM, Chen X, Li JY, Weiss S, Mishra J, Cheema FH, Markowitz G, Suganami T, Sawai K, Mukoyama M, Kunis C, D'Agati V, Devarajan P, Barasch J. Endocytic delivery of lipocalin-siderophore-iron complex rescues the kidney from ischemia-reperfusion injury. *J Clin Invest.* 2005,115:610-21.
6. Mishra J, Dent C, Tarabishi R, Mitsnefes MM, Ma Q, Kelly C, Ruff SM, Zahedi K, Shao M, Bean J, Mori K, Barasch J, Devarajan P. Neutrophil gelatinase-associated lipocalin (NGAL) as a biomarker for acute renal injury after cardiac surgery. *Lancet.* 2005,365:1231-8.
7. Nickolas TL, O'Rourke MJ, Yang J, Sise ME, Canetta PA, Barasch N, Buchen C, Khan F, Mori K, Giglio J, Devarajan P, Barasch J. Sensitivity and specificity of a single emergency department measurement of urinary neutrophil gelatinase-associated lipocalin for diagnosing acute kidney injury. *Ann Intern Med.* 2008,148:810-19.
8. Paragas N, Qiu A, Zhang Q, Samstein B, Deng SX, Schmidt-Ott KM, Viltard M, Yu W, Forster CS, Gong G, Liu Y, Kulkarni R, Mori K, Kalandadze A, Ratner AJ, Devarajan P, Landry DW, D'Agati V, Lin CS, Barasch J. The Ngal reporter mouse detects the response of the kidney to injury in real time. *Nat Med.* 2011,17:216-22.
9. Flo TH, Smith KD, Sato S, Rodriguez DJ, Holmes MA, Strong RK, Akira S, Aderem A. Lipocalin 2 mediates an innate immune response to bacterial infection by sequestering iron. *Nature.* 2004,432:917-21.
10. Yang J, Goetz D, Li JY, Wang W, Mori K, Setlik D, Du T, Erdjument-Bromage H, Tempst P, Strong R, Barasch J. An iron delivery pathway mediated by a lipocalin. *Mol Cell.* 2002,10:1045-56.
11. Kuwabara T, Mori K, Mukoyama M, Kasahara M, Yokoi H, Saito Y, Yoshioka T, Ogawa Y, Imamaki H, Kusakabe T, Ebihara K, Omata M, Satoh N, Sugawara A, Barasch J, Nakao K. Urinary neutrophil gelatinase-associated lipocalin levels reflect damage to glomeruli, proximal tubules, and distal nephrons. *Kidney Int.* 2009,75:285-94.
12. Bolignano D, Lacquaniti A, Coppolino G, Donato V, Campo S, Fazio MR, Nicocia G, Buemi M. Neutrophil gelatinase-associated lipocalin (NGAL) and progression of chronic kidney disease. *Clin J Am Soc Nephrol.* 2009,4:337-44.

13. Xu SY, Pauksen K, Venge P: Serum measurements of human neutrophil lipocalin (HNL) discriminate between acute bacterial and viral infections. *Scand J Clin Lab Invest.* 1995,55:125-31.
14. Martensson J, Bell M, Oldner A, Xu S, Venge P, Martling CR. Neutrophil gelatinase-associated lipocalin in adult septic patients with and without acute kidney injury. *Intensive Care Med.* 2010,36:1333-40.
15. Kjeldsen L, Johnsen AH, Sengelov H, Borregaard N. Isolation and primary structure of NGAL, a novel protein associated with human neutrophil gelatinase. *J Biol Chem.* 1993,268:10425-32.
16. Rudd PM, Mattu TS, Masure S, Bratt T, Van den Steen PE, Wormald MR, Küster B, Harvey DJ, Borregaard N, Van Damme J, Dwek RA, Opdenakker G. Glycosylation of natural human neutrophil gelatinase B and neutrophil gelatinase B-associated lipocalin. *Biochemistry.* 1999,38:13937-50.
17. Kanda J, Mizumoto C, Kawabata H, Tsuchida H, Tomosugi N, Matsuo K, Uchiyama T. Serum hepcidin level and erythropoietic activity after hematopoietic stem cell transplantation. *Haematologica.* 2008,93:1550-4.
18. Yamanaka R, Barlow C, Lekstrom-Himes J, Castilla LH, Liu PP, Eckhaus M, Decker T, Wynshaw-Boris A, Xanthopoulos KG. Impaired granulopoiesis, myelodysplasia, and early lethality in CCAAT/enhancer binding protein epsilon-deficient mice. *Proc Natl Acad Sci U S A.* 1997,94:13187-92.
19. Radoska HS, Huettner CS, Zhang P, Cheng T, Scadden DT, Tenen DG. CCAAT/enhancer binding protein alpha is a regulatory switch sufficient for induction of granulocytic development from bipotential myeloid progenitors. *Mol Cell Biol.* 1998,18:4301-14.
20. Chumakov AM, Grillier I, Chumakova E, Chih D, Slater J, Koeffler HP. Cloning of the novel human myeloid-cell-specific C/EBP-epsilon transcription factor. *Mol Cell Biol.* 1997,17:1375-86.
21. Axelsson L, Bergenfeldt M, Ohlsson K. Studies of the release and turnover of a human neutrophil lipocalin. *Scand J Clin Lab Invest.* 1995,55:577-88.
22. Gombart AF, Kwok SH, Anderson KL, Yamaguchi Y, Torbett BE, Koeffler HP. Regulation of neutrophil and eosinophil secondary granule gene expression by transcription factors C/EBP epsilon and PU.1. *Blood.* 2003,101:3265-73.
23. Saiga H, Nishimura J, Kuwata H, Okuyama M, Matsumoto S, Sato S, Matsumoto M, Akira S, Yoshikai Y, Honda K, Yamamoto M, Takeda K. Lipocalin 2-dependent inhibition of mycobacterial growth in alveolar epithelium. *J Immunol.* 2008,181:8521-27.
24. Cai L, Rubin J, Han W, Venge P, Xu S. The origin of multiple molecular forms in urine of HNL/NGAL. *Clin J Am Soc Nephrol.* 2010,5:2229-35.
25. Cowland JB, Borregaard N. The individual regulation of granule protein mRNA levels during neutrophil maturation explains the heterogeneity of neutrophil granules. *J Leukoc Biol.* 1999,66:989-95.

26. Nickolas TL, Forster CS, Sise ME, Barasch N, Valle DS, Viltard M, Buchen C, Kupferman S, Carnevali ML, Bennett M, Mattei S, Bovino A, Argentiero L, Magnano A, Devarajan P, Mori K, Erdjument-Bromage H, Tempst P, Allegri L, Barasch J. NGAL (Lcn2) monomer is associated with tubulointerstitial damage in chronic kidney disease.
27. Yan L, Borregaard N, Kjeldsen L, Moses MA. The high molecular weight urinary matrix metalloproteinase (MMP) activity is a complex of gelatinase B/MMP-9 and neutrophil gelatinase-associated lipocalin (NGAL). Modulation of MMP-9 activity by NGAL. *J Biol Chem.* 2001;276:37258-65.

Figure Legends

Fig. 1 Changes of blood neutrophil counts, serum and urinary LCN2 levels in Groups 1 and 2.

Serum and urine (s, u)LCN2 levels and blood neutrophil counts are expressed as median (interquartile range). AKI, acute kidney injury. * $p < 0.05$ vs. Pre-transplantation (Pre); # $p < 0.05$ vs. Group 1 at 3 weeks.

Fig. 2 Changes of blood neutrophil counts and serum LCN2 levels in Groups 3 and 4 (G3, G4).

Serum (s)LCN2 levels and neutrophil counts are expressed as median (interquartile range) for Group 4. For Group 3, values of two subjects are shown separately. Urinary LCN2 concentrations were highly variable among cases in Groups 3 and 4, and are not presented here (see online supplementary Fig. S2). sLCN2 in Group 4 at 3 weeks was 305 (147-1074) ng/ml and the top error bar for interquartile range was larger than what is shown in this figure (as indicated in dotted line). * $p < 0.05$ vs Pre-transplantation (Pre).

Fig. 3 Correlation of blood neutrophil counts and serum LCN2 levels.

Closed circles and a solid line, non-AKI points and their regression line ($r = 0.73$, $p < 0.001$); open circles and a dotted line, AKI points and their regression line ($r = 0.35$, $p = 0.12$).

Fig. 4 Relative ratios of high- and low-molecular-weight LCN2 in the serum and urine.

a Serum LCN2 (sLCN2) levels. Values are mean \pm SEM. $n = 10$, 4 and 5 from the left. **b** Urinary LCN2 (uLCN2) levels. SCT, patients undergoing stem cell transplantation who either had or did not have AKI ($n = 2$ each, mean of 4 is shown). SCT+Proteinuria, SCT patients who developed AKI with overt proteinuria (> 1 g/g creatinine, mean of $n = 2$ is shown). Cases of focal segmental glomerulosclerosis (FSGS; $n = 1$) and diabetic nephropathy (DN; mean of $n = 2$) who had overt proteinuria are also shown. Their clinical data are available in online suppl. fig. 3.

Fig. 5 C/EBP ϵ -dependent LCN2 expression.

a Effects of C/EBP α and ϵ overexpression upon human LCN2 promoter activity. Values are mean \pm SEM. $n = 4$. * $p < 0.05$ vs control. **b** Serum total Lcn2 levels including both HMW and LMW forms were measured by ELISA in C/EBP ϵ knockout (KO), heterozygous (Het) and WT (wild-type) mice. $n = 4$. **c** Western blot analysis of serum LMW Lcn2 from C/EBP ϵ KO and WT mice (#1 to 3, respectively, $n = 3$). Serum aliquots (2.5 or 10 μ l) from YM-100 flow-through were separated by SDS-PAGE in reducing conditions. rmLcn2, recombinant mouse Lcn2 as standards. **d** Relative ratios of high- and low-molecular-weight Lcn2 in the serum of C/EBP ϵ KO and WT mice. $n = 4$.

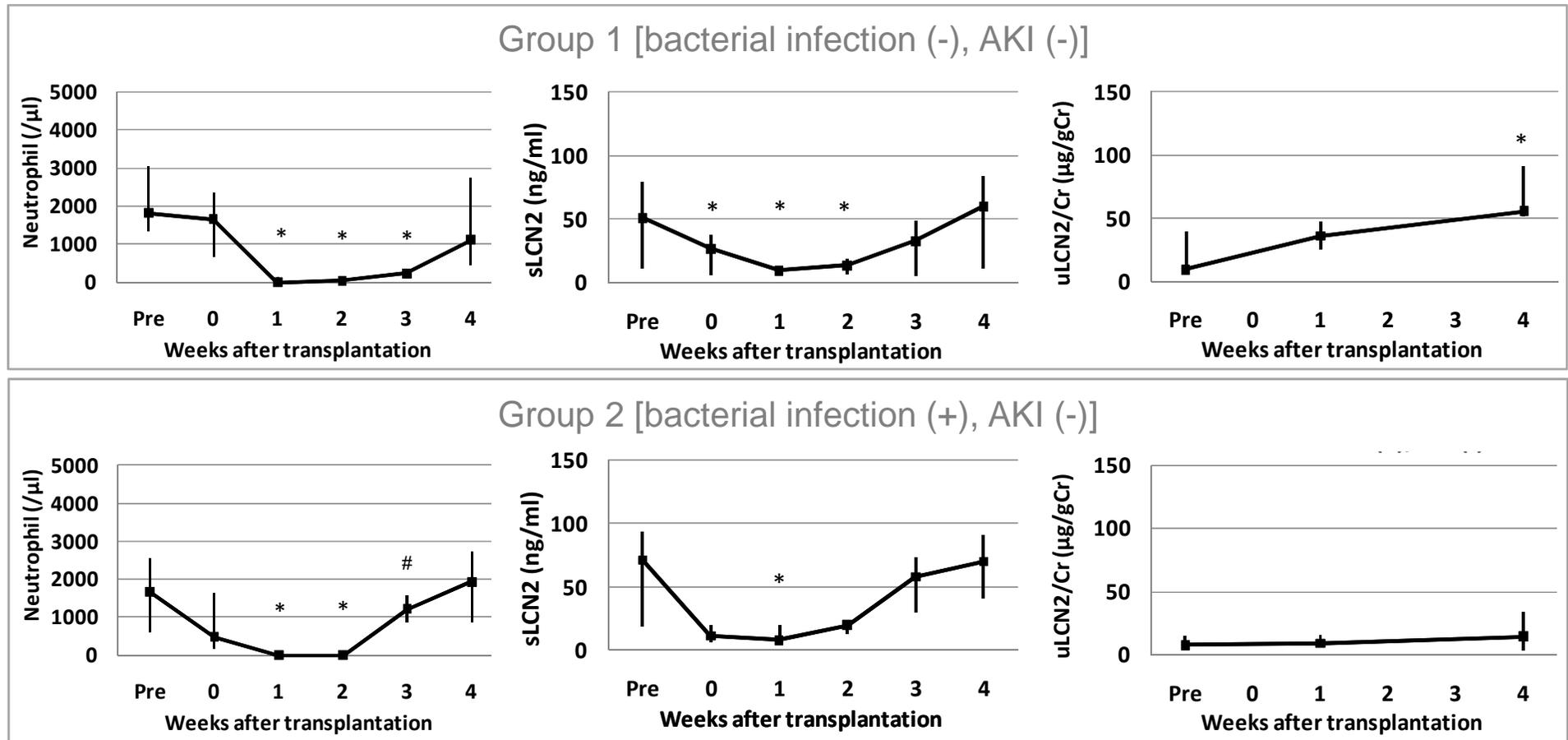


Fig. 1

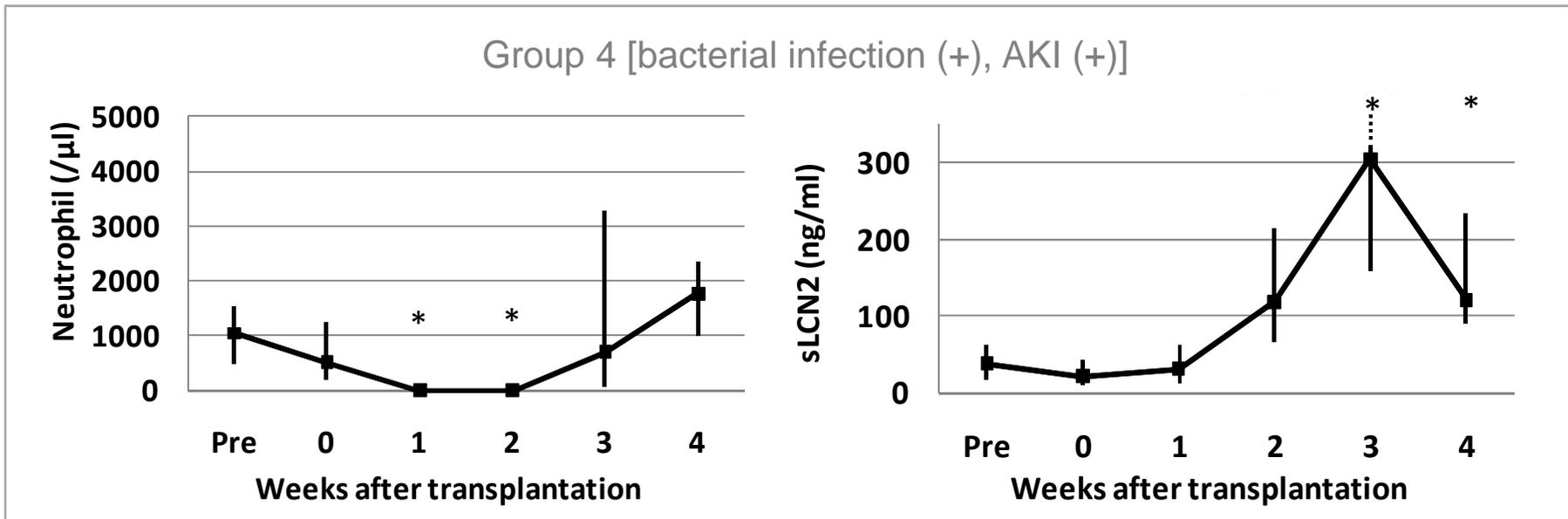
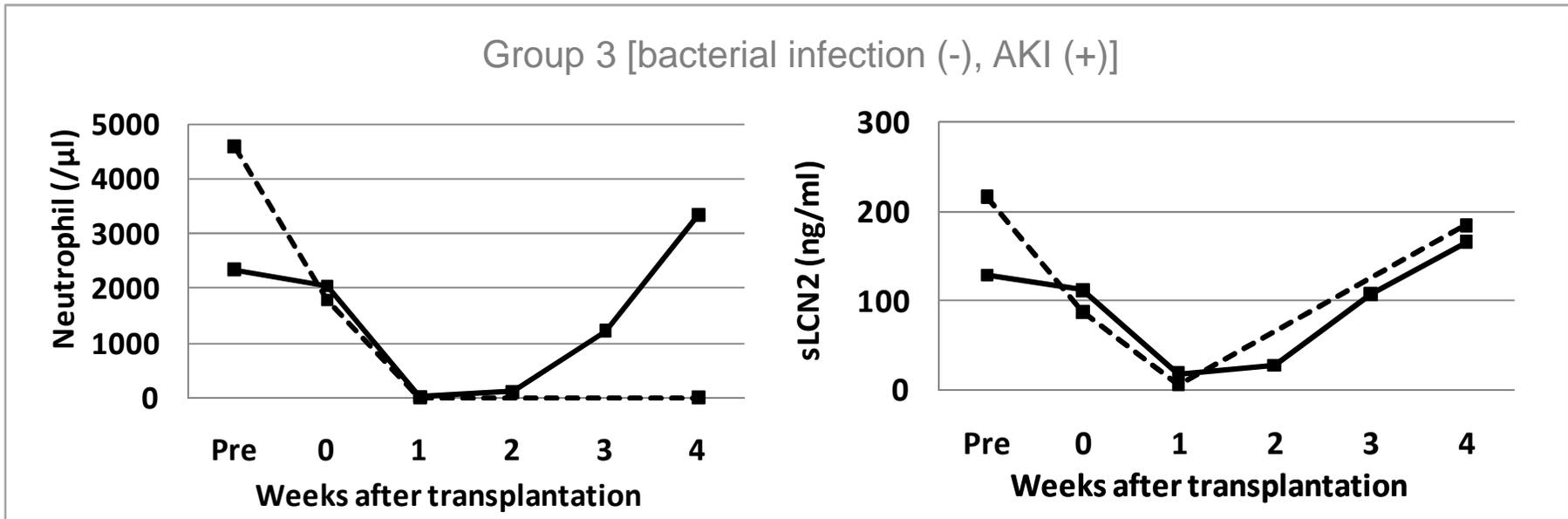
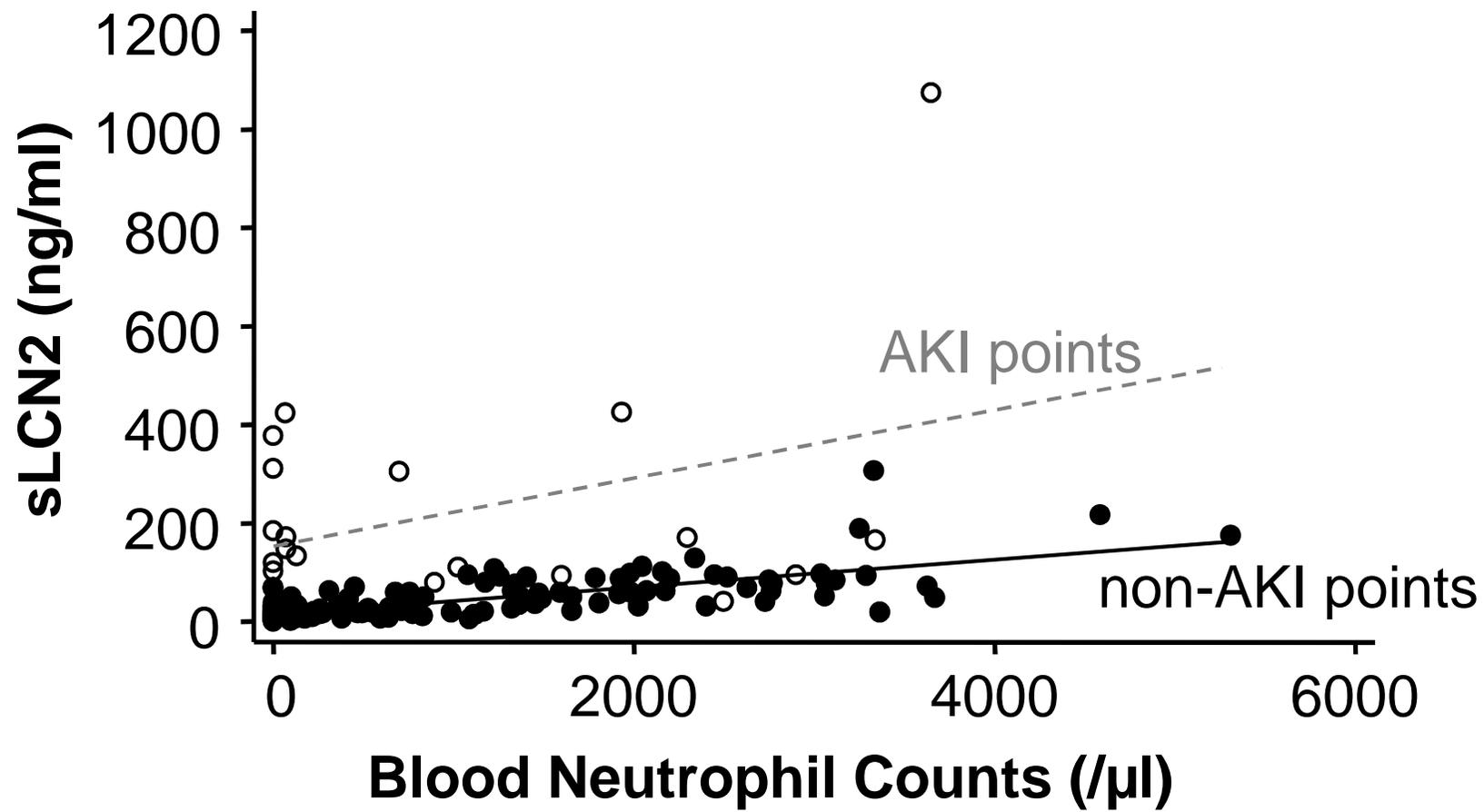


Fig. 2



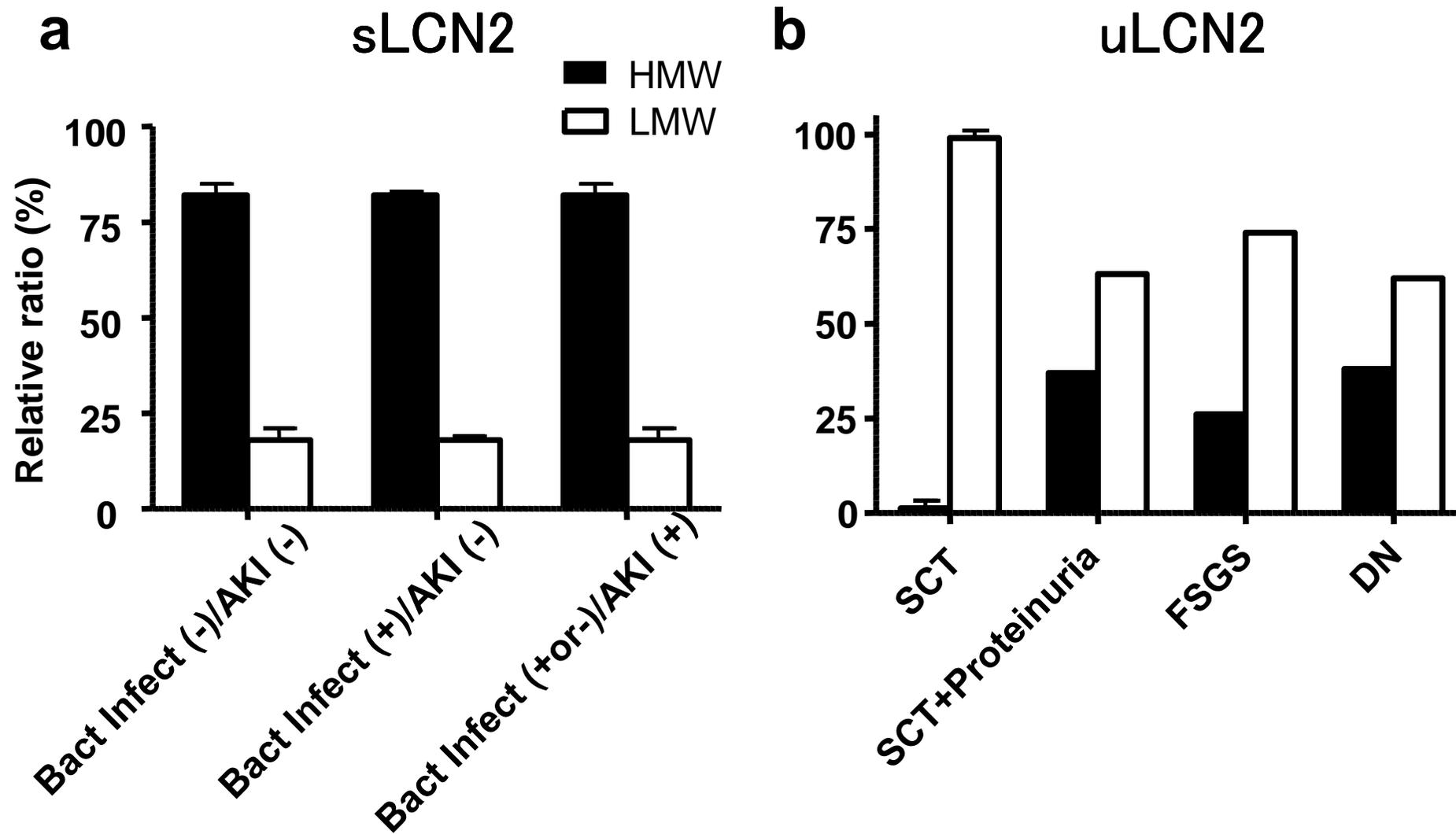


Fig. 4

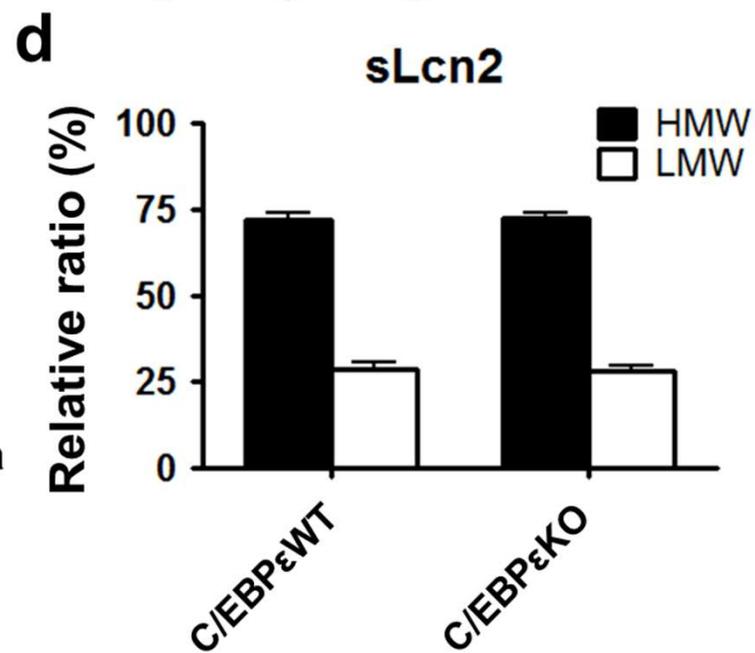
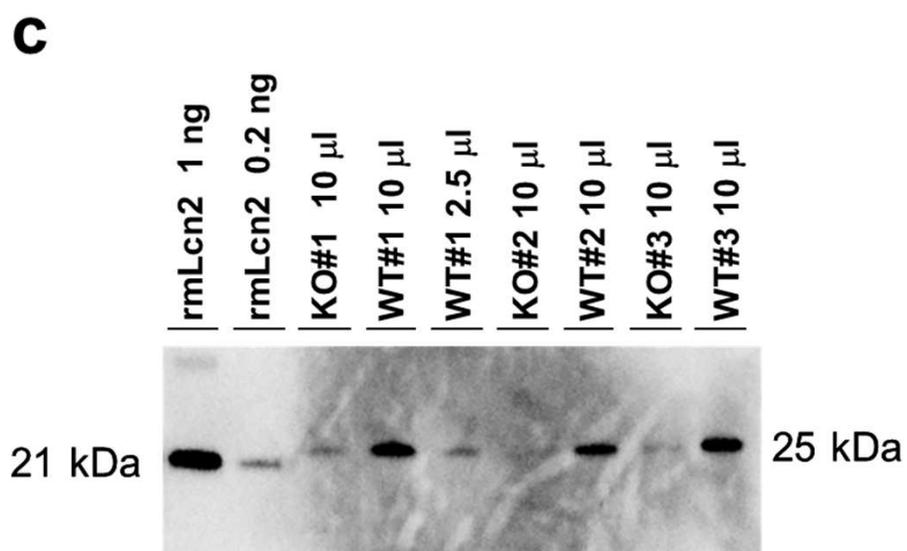
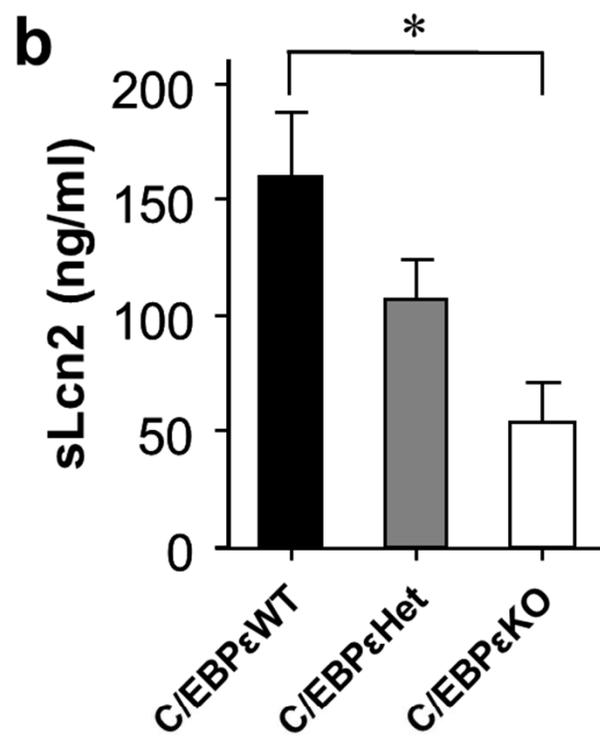
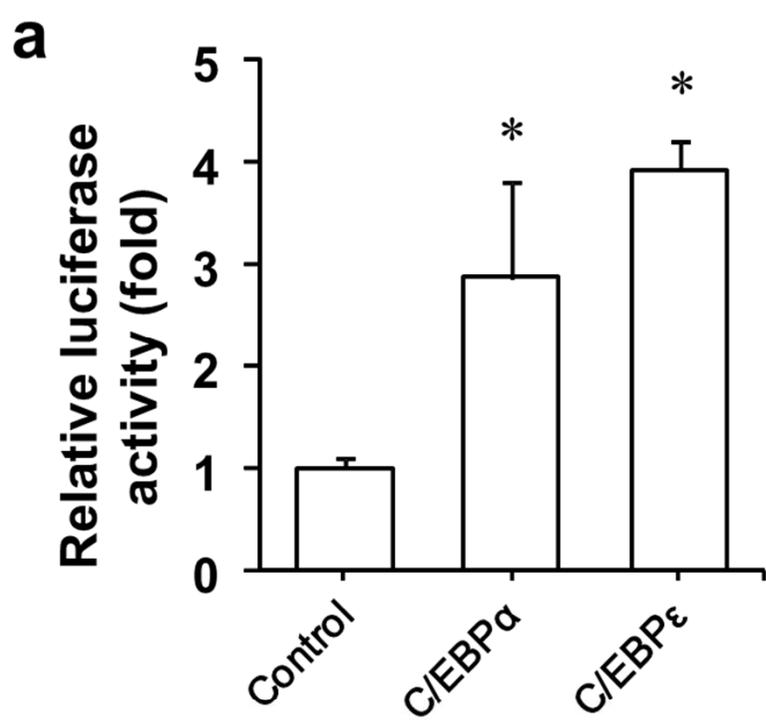


Fig. 5

Table 1 Categorization of patients who underwent SCT

Group	Bact Infect	AKI	Number	Peak sCRP (mg/dl)	Peak sCr (mg/dl)
1	-	-	12	0.8 (0.3-4.4)	0.8 (0.6-1.1)
2	+	-	12	7.0 (2.8-11.1)*	0.8 (0.6-0.9)
3	-	+	2	3.0 [1.0-4.9] [#]	2.4 [1.0-3.7] [#]
4	+	+	10	17.1 (5.4-24.9)*	2.3 (1.0-3.4)*

Bact Infect, bacterial infection; AKI, acute kidney injury;

Peak, peak value during observation period (which is within 4 weeks after SCT);

sCRP, serum C-reactive protein; sCr, serum creatinine.

Values are median (interquartile range), or mean [range][#].

*p < 0.05 vs Group 1.

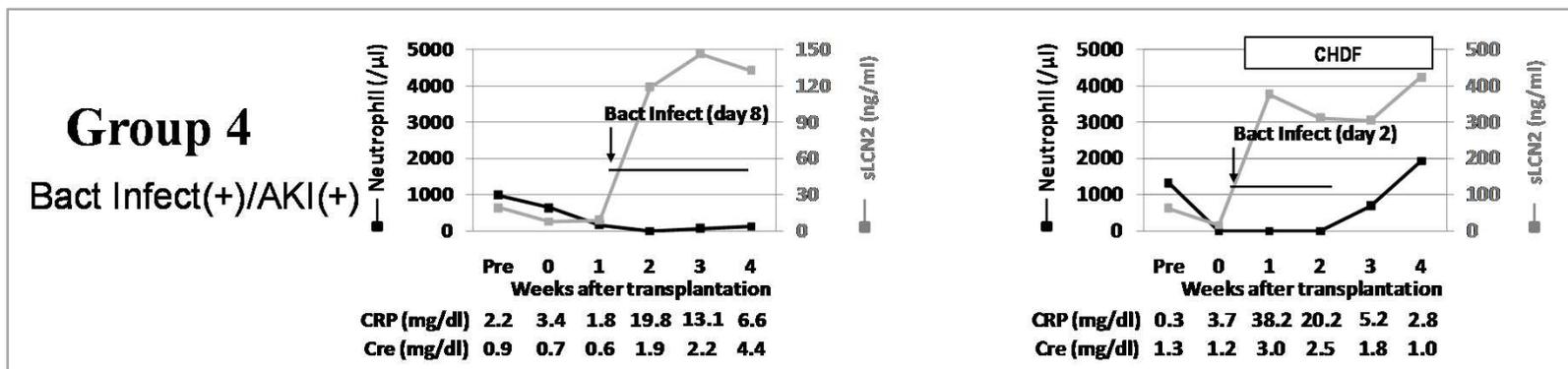
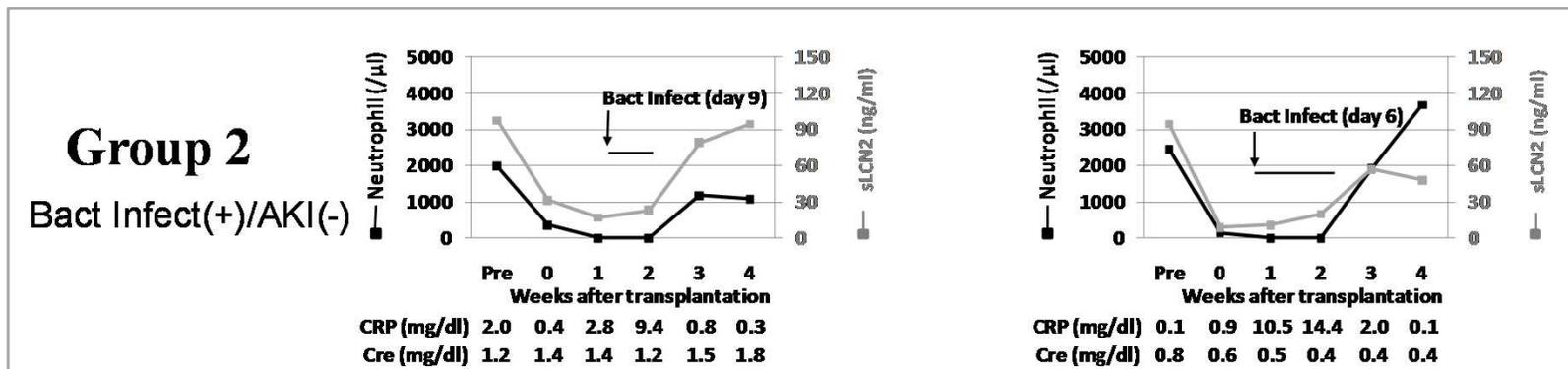
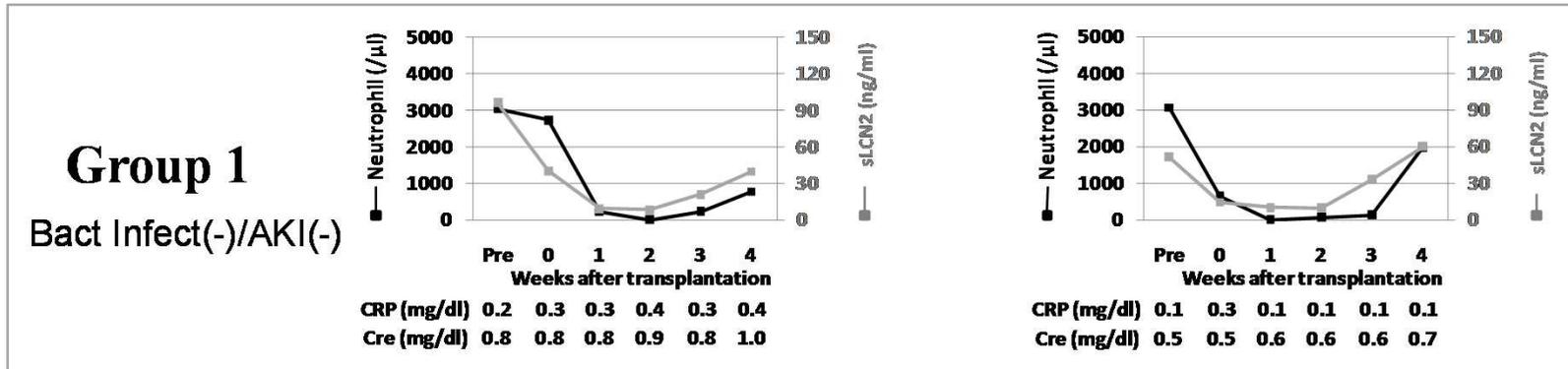
Table 2 Correlation of serum LCN2 levels with clinical parameters

Independent variables	Univariate		Multivariate	
	Standardized coefficient	p	Standardized coefficient	p
Neutrophil counts	0.570	<0.001	0.574	<0.001
Lymphocyte counts	-0.146	0.064		
Platelet counts	-0.049	0.525		
Hemoglobin	0.003	0.970		
Creatinine	0.217	0.005	0.100	0.160
CRP	0.233	0.002	0.159	0.031
BMI	0.190	0.093		

CRP, C-reactive protein. BMI, body mass index.

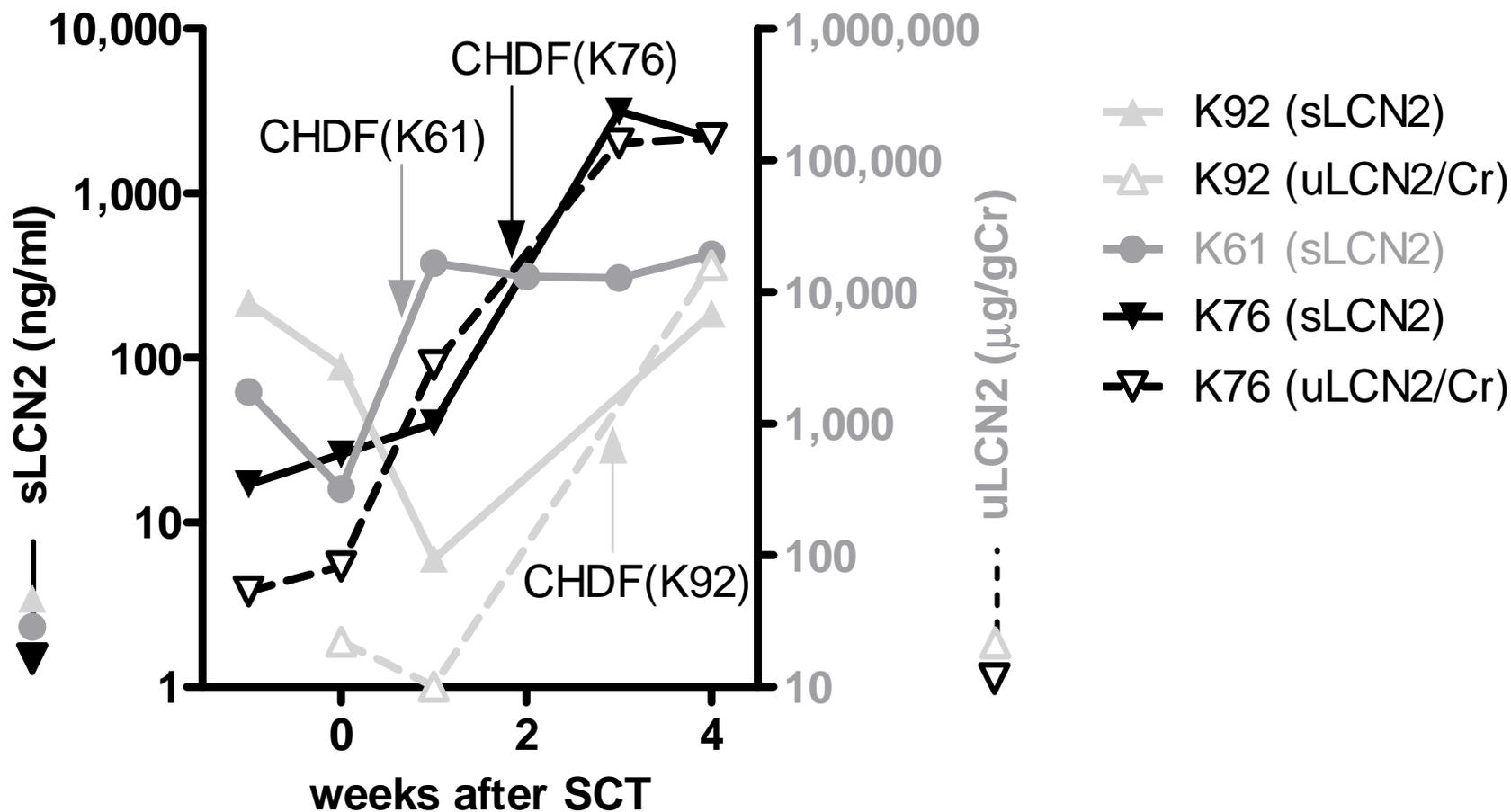
Supplementary Fig. S1

Clinical courses of 2 representative cases in each group. Levels of blood neutrophil counts (black), serum LCN2 (gray), CRP and creatinine (Cre) are shown. Arrows and bars, onset (also indicated in parenthesis) and duration of bacterial infection (Bact Infect); box, period of continuous hemodiafiltration (CHDF).



Supplementary Fig. S2

Serum and urinary LCN2 levels in patients who received continuous hemodiafiltration (CHDF). Arrows indicate the points when CHDF was started. SCT, stem cell transplantation. At the first week, patient K61 (in Group 4) had neutrophil, $0/\mu\text{l}$; sCr, 3.0 mg/dl; sCRP, 38.2 mg/dl; sLCN2, 377 ng/ml. At the first week, case K76 (in Group 4) had neutrophil, $0/\mu\text{l}$; sCr, 1.4 mg/dl; sCRP, 3.4 mg/dl; uLCN2, 2782 $\mu\text{g/gCr}$. At the fourth week, case K92 (in Group 3) had neutrophil, $0/\mu\text{l}$; sCr, 3.7 mg/dl; sCRP, 1.0 mg/dl; uLCN2, 16000 $\mu\text{g/gCr}$.



Supplementary Fig. S3

Separation of samples into HMW and LMW fractions and LCN2 measurement. **a**

(a) Methods of serum and urine fractionation.

(i) 100 μ l aliquots of samples were centrifuged at 10,000 x g for 30 min through 100-kDa cutoff filter (YM-100).

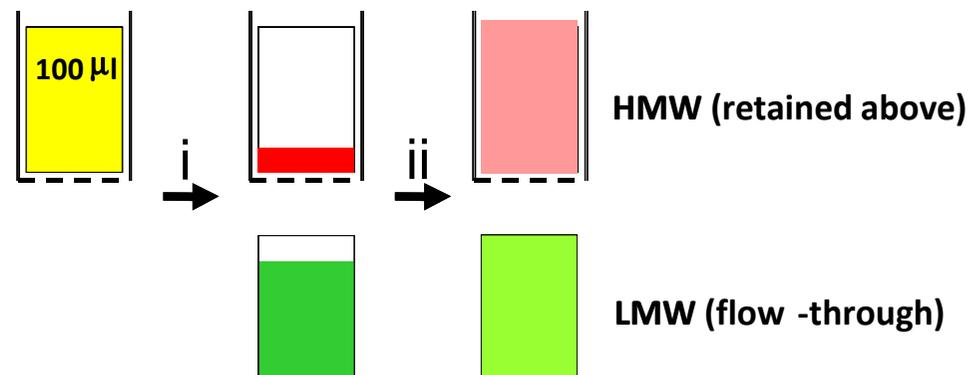
(ii) The solution retained above the membrane was recovered by inverting the column and centrifuging on top of a new tube for 2 min. The volumes of the flow-through and the remaining were adjusted back to 100 μ l by adding distilled water before analysis with LCN2 ELISA.

(b) Clinical variation and relative abundance of HMW and LMW LCN2 in each sample.

Values are mean \pm SEM [range].

Recovery of LCN2 immunoreactivity

[(HMW+LMW)/unfractionated] was $73 \pm 4\%$ (mean \pm SE, n = 19) for serum and $78 \pm 5\%$ (n = 9) for urine. SCT, stem cell transplantation. sCr, serum creatinine. sCRP, serum C-reactive protein. FSGS, focal segmental glomerulosclerosis. DN, diabetic nephropathy.



Category	Serum LCN2 Analysis		
	SCT	SCT	SCT
number	10	4	5
AKI	-	-	+
Infection	-	+	+ or -
WBC (/ul)	3390 \pm 743 [0-7400]	2325 \pm 1355 [0-5100]	580 \pm 555 [0-2800]
sCr (mg/dl)	0.9 \pm 0.1 [0.5-1.4]	0.9 \pm 0.1 [0.7-1.2]	2.3 \pm 0.4 [1.4-3.7]
sCRP (mg/dl)	1.3 \pm 1.0 [0.1-2.8]	12.9 \pm 6.2 [4.9-31.2]	13.7 \pm 7.1 [1.0-39.0]
HMW-LCN2 (relative %)	82 \pm 3 [64-96]	82 \pm 1 [79-83]	82 \pm 3 [75-91]
LMW-LCN2 (relative %)	18 \pm 3 [4-36]	18 \pm 1 [17-21]	18 \pm 3 [9-25]
Unfractionated LCN2 (ng/ml)	107 \pm 29 [17-306]	69 \pm 17 [23-107]	187 \pm 64 [40-424]

Category	Urinary LCN2 Analysis			
	SCT	SCT+Proteinuria	FSGS	DN
number	4	2	1	2
AKI	+ or -	+	-	-
Infection	+ or -	+ or -	-	-
WBC (/ul)	1100 \pm 662 [0-2800]	0 [0-0]	15400	5050 [4900-5200]
sCr (mg/dl)	1.5 \pm 0.5 [0.5-2.8]	2.6 [1.4-3.7]	0.6	1.4 [1.3-1.5]
sCRP (mg/dl)	18.9 \pm 9.6 [0.2-39.0]	2.2 [1.0-3.4]	0.1	0.6 [0.1-1.1]
HMW-LCN2 (relative %)	1.2 \pm 0.9 [0.2-4.0]	37 [35-40]	26	38
LMW-LCN2 (relative %)	99 \pm 1 [96-100]	63 [60-65]	74	62
Unfractionated LCN2 (ng/ml)	486 \pm 287 [46-1328]	5412 [4169-6655]	70	220 [169-270]

Supplementary Table S1

Diagnosis and treatment of patients who underwent SCT

Characteristics	n = 36
Recipient's age	54 (17-64)
Recipient's gender	
Male	16 [44%]
Female	20 [56%]
Diagnosis	
Leukemia	23 [64%]
Lymphoma	7 [19%]
Myelodysplastic syndromes	5 [14%]
Myeloma	1 [3%]
Disease status at transplant	
Complete remission	18 [50%]
Partial remission	1 [3%]
Not in remission	14 [39%]
Untreated	3 [8%]
Stem cell source	
Bone marrow	26 [72%]
Peripheral blood	2 [6%]
Cord blood	8 [22%]
Donor type	
Related donor	9 [25%]
Unrelated donor	18 [50%]
Unrelated cord blood unit	8 [22%]
Autologous	1 [3%]

Median (range) or patient number [%].

Supplementary Table S2

Measurement of LCN2/gelatinase B complex

Subject	K78			K86			Healthy1	Healthy2
Weeks after SCT	pre	+1	+4	pre	+1	+4		
AKI	-	-	-	-	-	+	-	-
Bact Infect	-	-	-	-	+	-	-	-
Serum								
LCN2 (ng/ml)	70	13	60	30	60	64	70	80
Complex (ng/ml)	7.8	0.8	7.5	7.2	0.8	4.1	15.9	29.7
Ratio of Complex (%)	10	6	11	19	1	6	19	27
Urine								
LCN2 (ng/ml)	46	57	20				4	20
Complex (ng/ml)	1.1	<0.3	<0.3				<0.3	<0.3

LCN2/gelatinase B (MMP-9) complex was measured by a Sandwich ELISA using two antibodies directed against LCN2 and gelatinase B, respectively (Quantikine, R&D Systems, Minneapolis, MN, USA).

LCN2 monomer preparation in BioPorto ELISA is not recognized by R&D Systems ELISA, since it does not contain gelatinase B epitope.

Standard preparation of LCN2/gelatinase B complex in R&D Systems ELISA (20 ng/ml) gave cross-activity of less than 1.5% in BioPorto ELISA (< 0.3 ng/ml).

Ratio of complex in the serum was calculated as complex/(LCN2+complex).