Circulating Neutrophil Gelatinase-Associated Lipocalin Level Is Determined by Nutritional Status and White Blood Cell Count but Not by Iron in Maintenance Hemodialysis Patients

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Running Title: Serum Ngal in Hemodialysis

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ABSTRACT

Neutrophil gelatinase-associated lipocalin (Ngal) is an iron-transporting factor which has a broad spectrum of activities including amelioration of kidney injury and host defense against pathogens. Its circulating concentrations are increased very rapidly in acute kidney injury (AKI), preceding the elevation of serum creatinine levels by a few days. In the present study, by cross-sectional analysis, we examined correlation of serum Ngal levels with standard laboratory findings and with parameters of nutritional and iron status among 79 maintenance hemodialysis (MHD) patients in two dialysis centers. Patients with infection, cancer or elevated C-reactive protein levels (> 0.5 mg/dl) were excluded. Univariate and multivariate linear regression analyses showed that the serum Ngal concentration was determined independently by % creatinine generation rate (an indicator of muscle mass), white blood cell (WBC) count, and anion gap (which likely reflects amount of food intake). Serum Ngal levels were decreased during hemodialysis session but only by 21%. None of the indices of iron metabolism, including blood hemoglobin, serum ferritin level and transferrin saturation were significantly associated with Ngal level. Furthermore, treatment of patients with intravenous iron injection caused elevation of ferritin, hemoglobin and iron-regulatory hormone hepcidin-25 levels, but Ngal levels were not affected. Retrospectively, patients who had admission because of infectious diseases within the past 2 years tended to exhibit lower serum Ngal levels compared to the rest. In conclusion, an AKI biomarker, serum Ngal level, appears to be regulated by muscle mass, nutrition, and WBC counts in MHD patients, independently of iron.

INTRODUCTION

Neutrophil gelatinase-associated lipocalin (Ngal) was intitinally purified from neutrophils with unknown function (Kjeldsen, JBC 1993). X-ray crystallography has revelaed that Ngal is bound to iron in the presence of organic cofactor which is called siderophore (Goetz, Mol Cell 2002). Thereafter, a number of iron-dependent Ngal activities have been identified (Mori and Nakao, Kidney Int 2007). As an iron donor, Ngal prevents kidney injury (Mori, JCI 2005), activates kidney differentiation, (Yang, Mol Cell 2002; Mori, Semin Cell Dev Biol 2003) and causes mesenchymal-epithelial transition of cancer cells (Hanai, JBC 2005). On the other hand, by iron chelation, Ngal exerts actions such as growth inhibition of pathogens including E. Coli (Flo, Nature 2004), Mycobacterium tuberculosis (Saiga, J Immunol 2008) and Klebsiella pneumoniae (Chan, J Immunol 2009), and apoptosis of pro-B cells (Devireddy, Cell 2005), erythroid progenitor cells (Miharada, FASEB J 2005) and microglia (Lee, Mori, J Immunol 2007). Insulin signalling is also positively or negatively modified by Ngal (Zhang, Mol Endocrinol 2008; Yang, Diabetes 2007). With respect to regulation of expression, inflammation (Cowland, J Immunol 2006), infection (Xu, Scand J Clin Lab Invest 1995), kidney injury (Mori, JCI 2005; Supavekin, KI 2003; Nickolas, AnnalsIM 2008; Mishra, Lancet 2005) and adipocyte differentiation (Yang, Diabetes 2007) are the major inducers of Ngal expression in epithelial and non-epihtelial cells, and the role of iron status itself in Ngal regulation remains largely unkown.

In acute kidney injury (AKI), serum and urinary Ngal levels are elevated rapidly, which occurs 1-3 days earlier than 50% increase in serum creatine levels above the baseline (Mishra, Lancet 2005; Wagener, Anesthesiology 2006). In this sense, by advocating 'Forest Fire Theory,' we have proposed that serum creatinine and glomerular filtration rate are indicators of previous injury (or results of functional loss), whereas blood and urinary Ngal may represent, at least partly, on-going renal damage (Mori and Nakao, KI 2007). Furthermore, in the settings of chronic kidney disease, Ngal level is correlated to serum creatinine (Bolignano, Kidney Blood Press Res 2008) and urinary protein levels (Ding, Clin Immunol 2007) and is potentially useful for the minitoring of treatment efficacy (Kuwabara, KI 2009; Kasahara, NDT 2009). However, very little is known about regulation of blood Ngal levels in end-stage renal disease (ESRD) patients. Patinets receiving maintenace hemodialysis (MHD) are a unique group of subjects whose cilinical parameters are closely monitored in routine practice. Furthermore, their dietary protein intake and muscle mass can be estimated by formulas using blood urea nitrogen (BUN) and creatinine levels before and after hemodialysis (HD) session (Borah, KI 1978; Shinzato, Artif Organs 1997), since urinary excretion of BUN and creatinine is almost negligible as compared to efficient clearance by HD. Here, we performed comprehensive analysis for the association of serum Ngal levels with 50 clinical parameters including iron status, nutrition, and history of admission in MHD patients. We also investigated the effects of iron administration and HD session upon serum Ngal levels.

RESULTS

Baseline Characteristics of Patients

Baseline characteristics of 79 out-patient maintenance hemodialysis (MHD) subjects are shown in Table 1. Of note, patients in Saiseikai Nakatsu Hospital had much longer HD period (dialysis center N, $n = 56$, median 87 months) as compared to Misugikai Satou Hospital (dialysis center M, $n = 23$, median 40 months, $P = 0.002$), which may have caused differences in several clinical parameters between the centers. Since inflammation, infection and malignancy have been already shown to increase serum Ngal levels (Cowland, J Immunol 2006; Xu, Scand J Clin Lab Invest 1995; Cho, J Histochem Cytochem 2009), we excluded 16 subjects (summarized in Supplementary Table 1) with infection, cancer or elevated C-reactive protein (CRP) levels (> 0.5 mg/dl)(Wang, Clin Chem 207), whose mean pre-dialysis serum Ngal levels were 27% higher compared to the rest of 79 patients ($P = 0.009$). Next, we studied variability of blood Ngal levels in 10 MHD patients by comparing 2 measurements which were 1 week apart (mean Ngal levels, 939 and 919 ng/ml, respectively). By paired analysis of the cases, the second measurement gave 99.6 ± 10.6% value (mean ± SD) of the first, indicating that serum Ngal levels were considerably stable, at least, for short term. Gender and presence of diabetes mellitus (DM) did not significantly affect mean serum Ngal levels (male, 940 ± 320, $n = 43$; female, 942 ± 321, $n = 36$; cases with DM, 902 ± 335, $n = 19$; without DM, 953 ± 314 ng/ml, $n = 60$).

Univariate Analysis of Serum Ngal Levels

By cross-sectional analysis, we studied association of pre-dialysis serum Ngal levels with 50 clinical parameters, which were laboratory data and indices of nutrition, iron status and HD efficiency, using univariate linear regression analysis (Table 2, Supplementary Table 2). Among the parameters examined, white blood cell (WBC) count, neutrophil count, serum creatinine, % creatinine generation rate (%CGR), anion gap, serum chloride (which had negative correlation), platelet count, and normalized protein catabolic rate (nPCR) were the top eight showing the strongest correlation coefficients with serum Ngal, which were either > 0.37 or < -0.37 ($P < 0.001$ for all). When 2 dialysis centers were analyzed separately, these 8 parameters were still significantly correlated with serum Ngal (Table 2). Indices of iron metabolism, including blood hemoglobin, hematocrit, mean corpuscular volume (MCV), reticulocyte count, serum ferritin, iron, total iron binding capacity (TIBC), unsaturated iron binding capacity (UIBC) and transferrin saturation (TSAT), were not significantly associated with Ngal level ($P > 0.05$ for all). Red blood cell (RBC) count showed a weak positive correlation with serum Ngal level in center N ($r = 0.32$, $P = 0.02$), but showed a tendency of negative correlation in center M ($r = -0.15$).

Multivariate Analysis of Serum Ngal Levels

We introduced the above 8 variables and investigated their correlation with serum Ngal levels. Multivariate linear regression analysis selected %CGR, WBC count and anion gap as independent variables determining serum Ngal levels (Table 3, Figure 1). These 3 variables explained 77% of the expected Ngal value. To categorize the 8 variables which were highly correlated to serum Ngal level, the association of these parameters to each other was examined. As shown in Table 4, %CGR, which is known as an indicator of muscle mass (Shinzato, Artif Organs 1997), exhibited strongest correlation to serum creatinine. WBC, neutrophil and platelet counts showed strong association to each other, which may represent hematopoietic activity of bone marrow. Anion gap and nPCR have been reported to be highly correlated to dietary protein intake (Dumler, ASAIO J 1999; Borah, KI 1978), and they were associated well to each other (Dumler, ASAIO J 1999). Consistently, serum Ngal levels were significantly correlated to markers of nutrition such as serum albumin ($r = 0.33$, $P = 0.003$), choline esterase ($r = 0.26$, $P = 0.02$) and phosphorus levels ($r = 0.28$, $P = 0.01$) by univariate analysis (Table 2). These findings suggest that muscle mass, nutrition and myeloid activity are the three major determinants of serum Ngal levels in MHD patients.

We further studied whether it is reasonable to dissect muscle mass (%CGR) and nutrition (nPCR) as independent clinical parameters in these patients (Figure 2), since they were significantly associated to each other (Table 4). One patient plotted at the end of left-top corner (corresponding to low %CGR and high nPCR) had rheumatoid arthritis and muscle atrophy which may have been caused by immobilization and by steroid-induced muscle catabolism. When average serum Ngal levels and blood WBC counts in quadrants (based upon %CGR and nPCR) were compared, the rank orders were different. These findings support that serum Ngal and WBC count are independent, and %CGR and nPCR are independent parameters, respectively, among MHD patients studied in this work.

Impact of HD Efficiency, HD Period and Age upon Serum Ngal

Since serum Ngal and creatinine levels were highly correlated ($r = 0.62$, $P < 0.001$, Table 2), we examined whether elevated Ngal was caused by low efficiency in HD. Oppositely, HD efficiency calculated as single-pool Kt/V (Kt/Vsp) was positively associated with Ngal levels ($r = 0.32$, $P = 0.004$, Figure 3). Furthermore, serum Ngal levels were negatively correlated to age ($r = -0.34$, $P = 0.002$) and positively correlated to HD period ($r = 0.34$, $P = 0.002$), suggesting that patients with long HD history contained substantial amount of young subjects whose nutritional conditions were good.

Impact of Iron Administration upon Serum Ngal

To directly test whether iron metabolism is linked to Ngal regulation, we examined the effects of iron administration in 6 MHD patients who had low serum ferritin levels (33 $\pm$ 20 ng/ml). After 10 doses of 50 mg intravenous iron injection (twice a week for 5 weeks), serum ferritin, blood hemoglobin and hepcidin-25 levels were significantly elevated at 8 weeks (Figure 4). Hepcidin-25 is a circulating hormone which inhibits intestinal absorption of iron and release of iron from macrophages (Nemeth, Science 2004; Tomosugi, Blood 2006). By contrast, serum Ngal levels were not affected during the observation period, indicating that abundance of systemic iron does not directly control serum Ngal levels.

Ngal Clearance by HD Session

Ngal is a 25-kDa secretory protein as a monomer but it may exist as larger molecule forms, such as Ngal:matrix metalloproteinase-9 (MMP-9) heterodimer (130 kDa)(Kjeldsen, JBC 1993). We examined whether circulating Ngal is removed by HD procedure. As shown in Figure 5, serum Ngal level showed a rapid decrease at 10 min (by 22%, $n = 4$, $P = 0.13$) after initiation of HD, which may be caused by absorption of Ngal to HD membrane and circuit, and it remained considerably stable during the rest of time in HD. At the end of HD, Ngal levels were decreased by 21 ± 14% ($n = 79$, $P < 0.001$ comparing before and after HD, Supplementary Table 1). As potential sources of circulating Ngal, we investigated the kinetics of blood WBC and neutrophil counts, and observed temporal drop at 10 min after initiation of HD but it recovered to baseline by 60 min (Craddock, JCI 1997), indicating that neutrophil count alone cannot explain the alteration of serum Ngal levels during HD session. In comparison, the arterial blood concentrations of low molecular weight (MW) substances such as potassium ion (MW 39 Dalton), BUN (60), creatinine (113), β2-microglobulin (12 kDa) were decreased constantly during HD session. On the other hand, albumin (60 kDa) and IgG (150 kDa) levels increased slightly as water removal proceeded during HD. The kinetics of arterial Ngal level was intermediate of low and high molecular weight substances, suggesting that Ngal was constantly removed during HD but by low efficiency.

Ngal Clearance by Renal Circulation

To study whether circulating Ngal is removed during renal passage, we collected plasma (instead of serum) from aorta and renal vein in a separate group of 30 patients who underwent coronary angiography at Nara Medical University Hospital. As shown in Table 5, in 15 patients without HD, plasma creatinine levels in renal vein were 19 ± 10% less compared to those in aorta ($P < 0.001$), and plasma Ngal levels in renal vein were 13 ± 12% less as compared to aorta ($P = 0.001$). On the other hand, in 15 MHD subjects, plasma creatinine and Ngal levels were not different between renal vein and aorta. As negative controls, plasma albumin and choline esterase concentrations in aorta and renal vein were similar both in non-HD and HD subjects. These findings suggest that circulating Ngal is, at least partly, either filtered, metabolized or degraded during renal passage in subjects with viable renal blood flow.

Infection and Serum Ngal

Ngal is an iron-chelating protein and suppresses growth of several important pathogens such as E. coli, Mycobacterium tuberculosis and Klebsiella pneumoniae (Flo, Nature 2004; Saiga, J Immunol 2008; Chan, J Immunol 2009). Furthermore, serum Ngal levels are markedly increased by bacterial infection (Xu, Scand J Clin Lab Invest 1995). To investigate functional significance of serum Ngal concentrations in MHD patients at stable, non-infected status, we retrospectively studied the history of admission during the past 2 years, and classified the causes as cardiovascular diseases, infectious diseases and others (Table 6). Of 79 patients, 42% had one or two admissions within the past 2 years. Average serum Ngal levels were not different in patients who had admission by all causes ($n = 33$) or by cardiovascular diseases ($n = 12$) as compared to patients without admission ($n = 46$). However, in 7 patients who had been admitted for the treatment of infection

(likely by bacteria), average serum Ngal levels tended to be lower (by 27%) compared to 46 admission-free patients (median 701 and 963 ng/ml, respectively), but the difference did not achieve statistical significance. At the same time, patients who had admission by infection had slightly lower mean WBC counts and nPCR values compared to all 79 subjects (5275 ± 1769 vs 5751 ± 1699 ng/ml, 0.82 ± 0.12 vs 0.91 ± 0.18 g/kg/day, respectively; not statistically significant). These findings imply that relatively low serum Ngal levels in a subset of MHD patients might have predisposed them to developing infectious disease.

Analysis without Excluded Cases

So far, among 95 patients enrolled, we have selected and analyzed 79 subjects who had low serum CRP levels (≤ 0.5 mg/dl), having neither infection nor cancer (Supplementary Table 1). To rule out possible selection bias, we investigated the data from all 95 patients, and again %CGR (r = 0.47), WBC count (r = 0.63) and anion gap (r = 0.52) were highly correlated to serum Ngal levels by univariate analysis (P < 0.001 for all). On the other hand, serum iron (r = -0.09, P = 0.39), ferritin (r = 0.12, P = 0.26) and TSAT (r = -0.08, P = 0.47) were not significantly correlated to serum Ngal. Furthermore, serum CRP level was significantly correlated to serum Ngal level when non-selected 95 patients were analyzed (r = 0.26, P = 0.01), but serum CRP level was not correlated to serum Ngal in 79 selected patients (r = 0.11, P = 0.33, Supplementary Table 2). We carried out multivariate analysis of serum Ngal level in all 95 patients using variables of CRP and above described 8 parameters (indicated by underlines in Table 2) and found that WBC, CRP, %CGR and anion gap were 4 independent determinants of serum Ngal level, explaining 78% of the expected value (Supplementary Table 3).

DISCUSSION

In the present study, using univariate and multivariate analyses, we show that serum Ngal levels in MHD patients are independenly determined by muscle mass (represented by %CGR), nutritional status (by anion gap) and WBC counts. By dot plot analysis of indices for muscle mass and nutritional status, it appears reasonable to recognize these two indices as independent clinical parameters. When patients with infection, cancer or elevated CRP levels are included in the analysis, CRP levels become additional determinant of serum Ngal concentrations.

In morbidly obese mice (Yan, Diabetes 2007) and humans (Wang, Clin Chem 2007), it was reported that serum Ngal levels are elevated but it was difficult to tell whether amount of food or amount of muscle mass plays a major role in their correlation. In the present study, we conclude that nutrition ($r = 0.54$ for anion gap) and physical constitution ($r = 0.58$ for %CGR) independently and potently affects circulating Ngal levels at least in MHD patients. Of note, neither pre- nor post-dialysis body mass index (BMI) is associated with pre-dialysis serum Ngal levels in MHD patients (Supplementary Table 2), likely because hypoalbuminuria or congestive heart failure causes inappropriate water retention and obesity-unrelated elevation of BMI. In obese mice, Ngal gene expression is enhanced in the liver and adipose tissue as compared to lean mice (Zhang, Mol Endocrinol 2008). Nutrional status is known to positively affect blood WBC count in humans (Drenick, Am J Clin Nutrition 1971). It remains elusive whether Ngal release from neutrophils is modulated by nutrition.

Metabolic acidosis (low pH) compensated by respiratory alkalosis (low pCO_2) is the typical pattern of acid-base balance in these 79 MHD patients. Metabolic acidosis associated with increased anion gap may be caused by underdialysis and anorexia. On the other hand, however, high dietary protein intake (and, therefore, high nPCR) may result in greater net acid gain and acidosis (thus, high anion gap), which is not necessarily harmful in well-dialyzed MHD patients (Dumler, ASAIO J 1999; Kalantar-Zadeh, Semin Dial 2004). In this work, serum Ngal levels are positively associated with anion gap, nPCR, and Kt/Vsp ($r = 0.32$, $P = 0.004$, Table 2; Bolignano, Nephrol Dial Transplant, in press), favoring the latter effect.

Since Ngal is an iron carrier protein (Goets, Mol Cell 2002; Mori, Kidney Int 2007), it is important to test whether iron status directly affects circulating Ngal concentrations. Here we show that serum Ngal levels are not significantly altered by repeated iron administration, amount of which is sufficient to cause 4.3-fold elevation in serum ferritin levels. Bolignano et al. recently reported that Ngal levels are elevated after iron injection in MHD patients but only by 9% (which was accompanied with 1.8-fold ferritin elevation)(Bolignano, Nephrol Dial Transplant, in press). Jiang reported that administration of iron or erythropoietin to mice does not change liver Ngal mRNA levels (Jiang, Blood Cells Mol Dis 2008). Taking these findings into account, we would like to conclude that iron is not a major determinant of serum Ngal levels, at least in MHD patients. Bolignano et al. also reported marginal correlation between serum Ngal and (log-transformed) TSAT levels ($r = 0.29$, $P = 0.04$) but it may be indirect effects as the authors suggested (Bolignano, Nephrol Dial Transplant, in press).

In the present study, we show that MHD patients with reduced serum Ngal levels tend to have a history of admission for the treatment of infectious diseases. These findings can be caused by reduced Ngal's bacteriostatic activity, or by accompanying malnutrition and neutropenia. Growth inhibition of several but not all pathogens by iron depletion is an established activity of Ngal (Flo, Nature 2004; Saiga, J Immunol 2008;

Chan, J Immunol 2009; Fluckinger, Antimicrob Agents Chemother 2004). Serum Ngal levels are 5-20 fold higher in MHD patients compared to normal subjects (Kuwabara, KI 2009), but iron-chelating activity of Ngal might be partly inhibited, since these patients are replete with exogenous iron to avoid anemia.

Circulating Ngal level is an early biomarker for AKI and high Ngal concentrations predict progression of renal damage (or serum creatinine elevation) and mortality in AKI (Dent, Crit Care 2007). On the other hand, we show here that high Ngal levels in MHD patients are closely associated with good nutritional status, and therefore potentially with reduced morbidity and mortality. These findings may, at first glance, appear confusing but similar paradoxical observation has been found for serum creatinine level, the gold standard for renal function evaluation, and homocysteine level as reverse epidemiology (Kalantar-Zadeh, KI 2003; JASN 2004).

In conclusion, here we show evidence indicating that nutrional status, muscle mass and WBC counts are important determinants of serum Ngal in MHD patients. It is likely that similar association also occurs in subjects with normal or nearly-normal renal function, just as serum creatinine is affected by muscle mass in patients with various renal functions. Since serum and urinary Ngal levels are now vigorously measured worldwide to evaluate a broad spectrum of kidney diseases, especially acute kidney injury, it is very important to elucidate the regulatory mechanism of Ngal concentration for precise interpretation of the results from Ngal measurements.

CONCISE METHODS

Patients

A total of 95 out-patient MHD subjects were recruited between December 2008 through March 2009 at two dialysis centers M and N in Osaka, Japan. Sixteen patients with infection, cancer or elevated serum CRP levels (> 0.5 mg/dl) were excluded in the primary analysis, until the overall investigation was carried out (Supplementary Tables 1 and 3). Patients were on HD for 3-4.5 h with type III or IV high-flux membranes three times a week. In 6 cases at center M who started to be treated with intravenous iron injection (Cideferron, macromolecular complex of ferric hydroxide with dextrin and citric acid; Nippon-zoki, Osaka, Japan), blood collection was carried out for 8 weeks. Presence of DM was defined by taking any oral anti-DM mediaction or insulin injection. A separate group of 30 patients were enrolled at Nara Medical University Hospital, who underwent elective coronary angiography for evaluation of ischemia heart disease (Iwama, J Am Coll Cardiol 2006). Only plasma was available for these patients. The protocol was approved by ethical committees in the above institutes and in Kyoto University Graduate School of Medicine (No. E-541). All the participants gave written informed consent.

Laboratory Analyses

Blood samples were collected at the beginning and end of HD session after the longest interdialytic period (on Monday or Tuesday). Serum was separated immediately and kept frozen at -80oC until analysis. Ngal was measured by sandwich ELISA (BioPorto; Gentofte,Denmark) usually after 4,000-fold dilution. Hepcidin-25 was determined at Medical Research Institute, Kanazawa Medical University by a proteomic method using surface-enhanced laser desorption ionization time of flight mass spectrometry (SELDI-TOF MS). Routine laboratory measurements were carried out in each clinical institute. Creatinine was measured with an enzymatic method.

Calculation of Clinical Indices

Calcium levels were normalized with Payne's formula only when serum albumin was less than 4 g/dl (Payne, Br Med J 1973). Anion gap was determined as $(Na^+ + K^+) - (Cl^- + HCO_3^-)$. Calculation as $Na^+ - Cl^- - HCO_3^-$ gave very similar findings (not shown). Kt/Vsp, nPCR and %CGR were determined by formulas previously described (Daugirdas, JASN 1993; Shinzato, Nephron 1994; Shinzato, Artif Organs 1997)

Statistical Analyses

All variables were expressed as mean ± SD or median (25 – 75 percentile). Difference in two groups was examined either by paired t test, or by Student's t test if Levene's F test assumed equal variance in the groups. When F test assumed unequal variance, Welch's test was carried out instead. Univariate linear regression analysis was performed with Spearman rank test. Multivariate linear regression analysis was done with stepwise method using entrance/exit tolerances of 0.05/0.10. All analyses were conducted using SPSS Version 17.0 (SPSS Inc., Chicago, IL, USA). The *P* values reported are two-sided and considered significant at < 0.05.

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DISCLOSURES

K. Mori and J. Barasch are co-inventors on the Ngal patent filed by Columbia University.

REFERENCES

FIGURES

Figure 1. Correlation of pre-dialysis serum Ngal levels with % creatinine generation rate (CGR), WBC count and anion gap. Linear line in each graph shows regression line. *n* = 79.

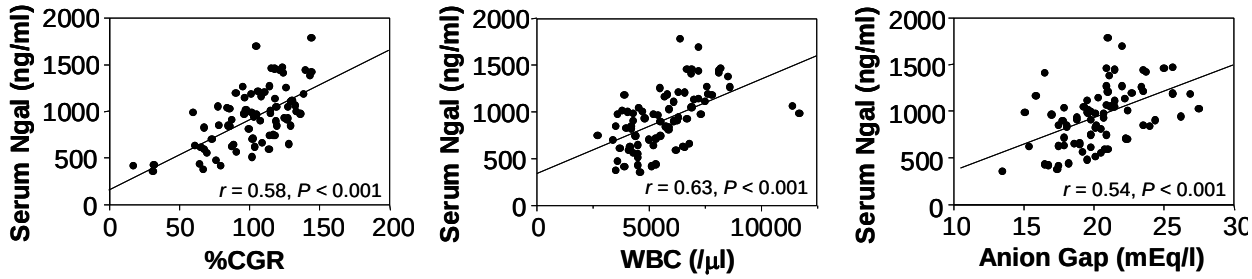

Figure 2. Distribution of %CGR and normalized protein catabolic rate (nPCR) in MHD patients. (A) Subjects were subdivided into quadrants (1st – 4th) based upon median values of %CGR and nPCR (solid lines). Regression line is shown as dotted line ($n = 79$, $r = 0.36$, $P = 0.001$). The patient plotted at the end of left-top corner had rheumatoid arthritis and muscle atrophy (arrow). (B) When mean (± SD) serum Ngal level for each quadrant was compared, the rank order was 1st quadrant (%CGR high, nPCR high) = 4th quadrant (high, low) ≥ 2nd quadrant (low, high) > 3rd quadrant (low, low). (C) On the other hand, the rank order of mean blood WBC count was 1st ≥ 2nd = 4th > 3rd quadrants. Mean values were compared by Tukey's test.

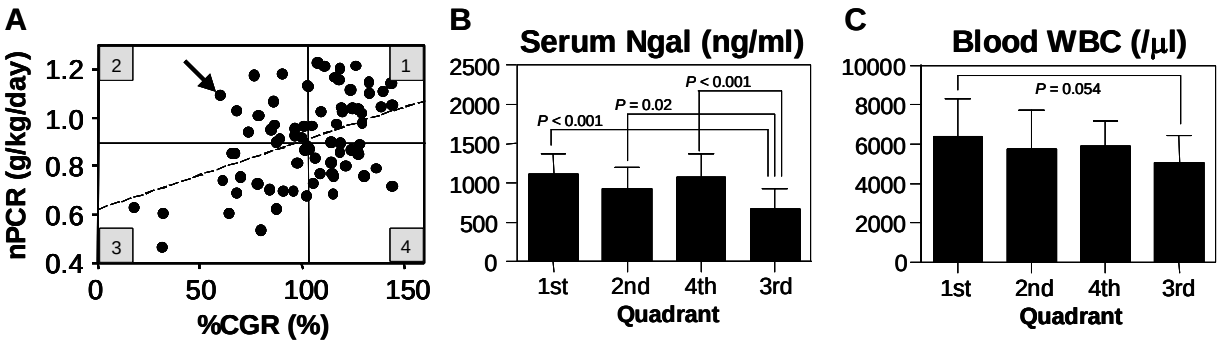

Figure 3. Correlation of serum Ngal level with serum creatine (Cr) level, single-pool Kt/V (Kt/Vsp), age and HD period. *n* = 79.

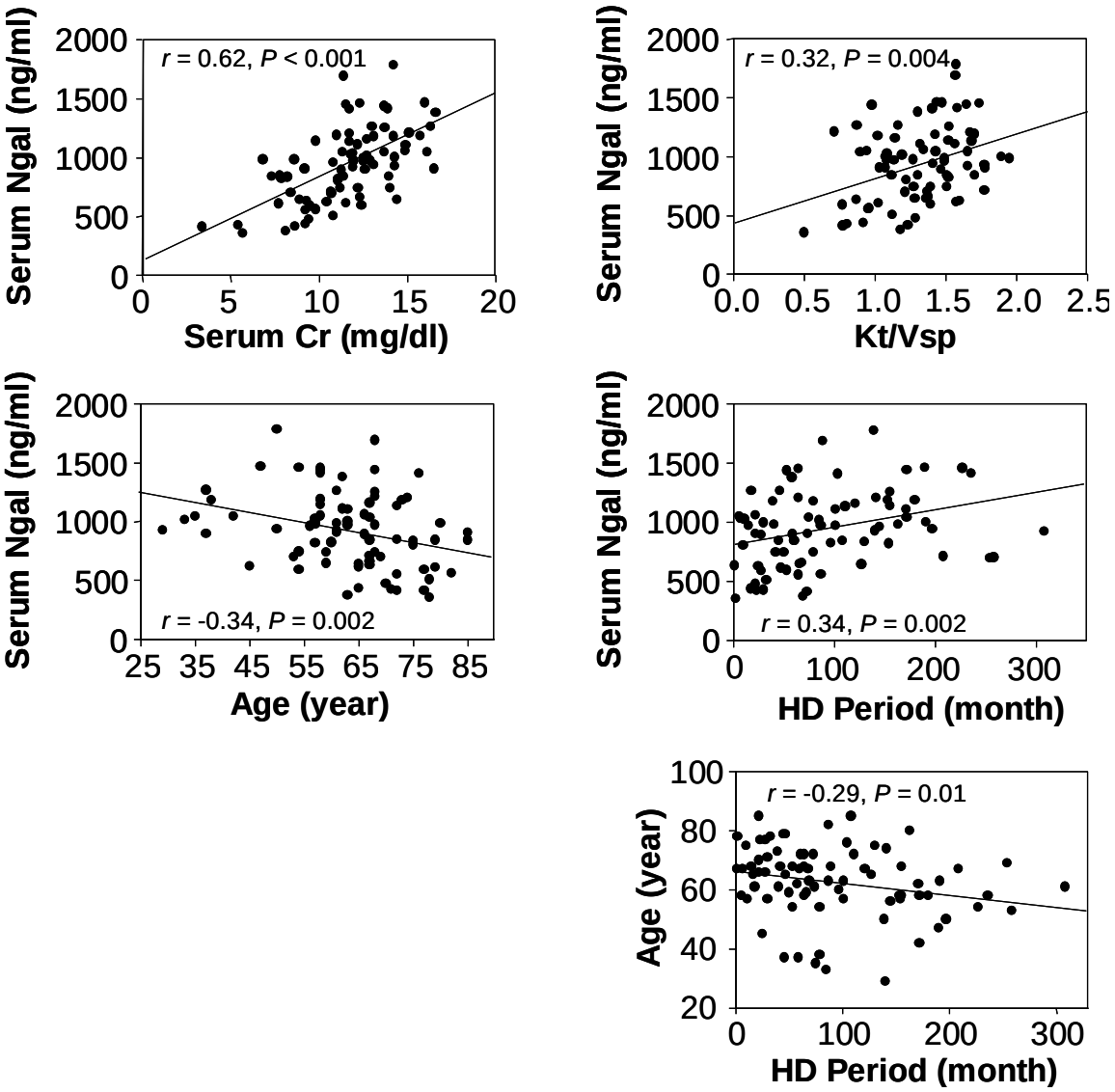

Figure 4. Effects of iron administration upon clinical parameters in MHD patients. Comparison between groups was carried out by paired t test. $n = 6$.

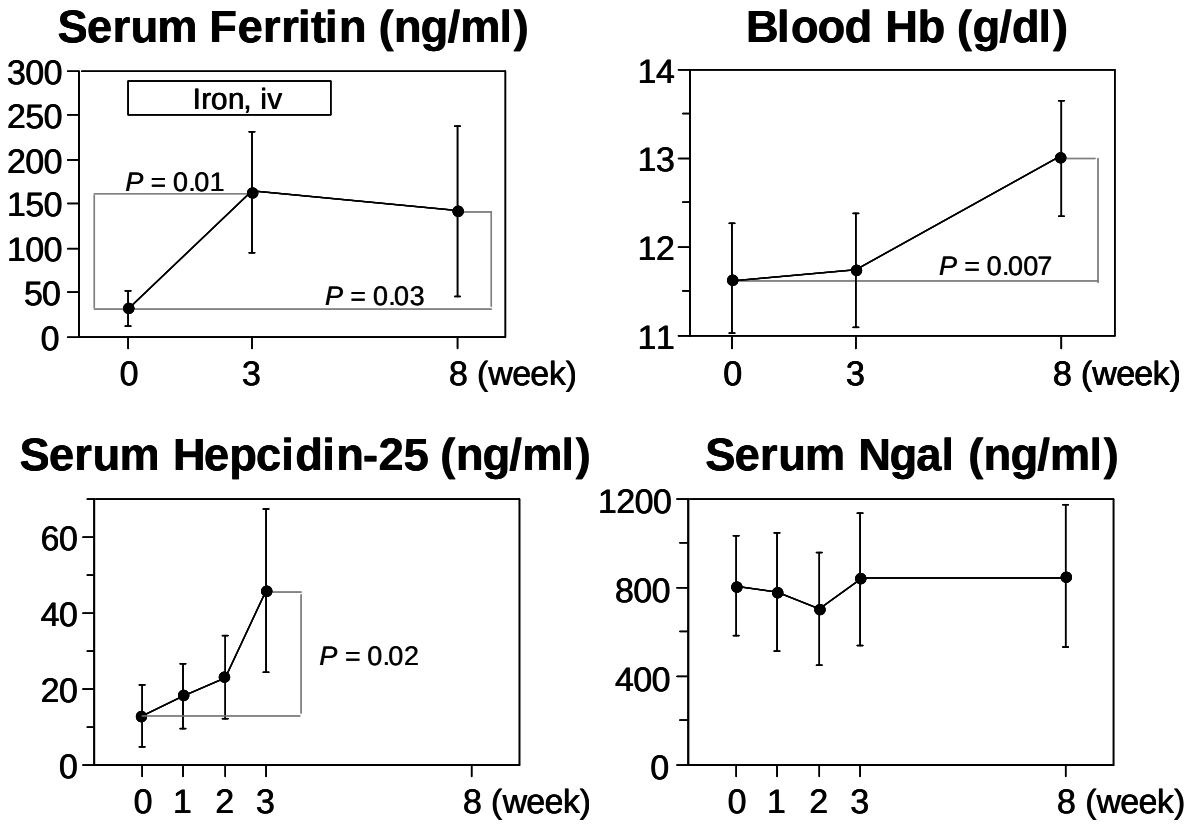

Figure 5. Removal of circulating substances by HD session. Blood was longitudinally drawn from the arterial and venous ends of HD circuit inserted into the internal shunt located at the forearm (closed circle and open box, respectively). Serum levels of K, BUN, creatinine (Cr), β2-microglobulin (MG), albumin, IgG and Ngal, as well as blood WBC and neutrophil counts were examined in 4 patients. Arterial levels of K, BUN, Cr, β2-MG were always higher than venous levels, indicating active removal by HD. On the other hand, venous levels of albumin and IgG tended to be higher than arterial levels, reflecting hemoconcentration. Comparison between groups was carried out by paired t test. *****, $P < 0.05$ between arterial and venous sides.

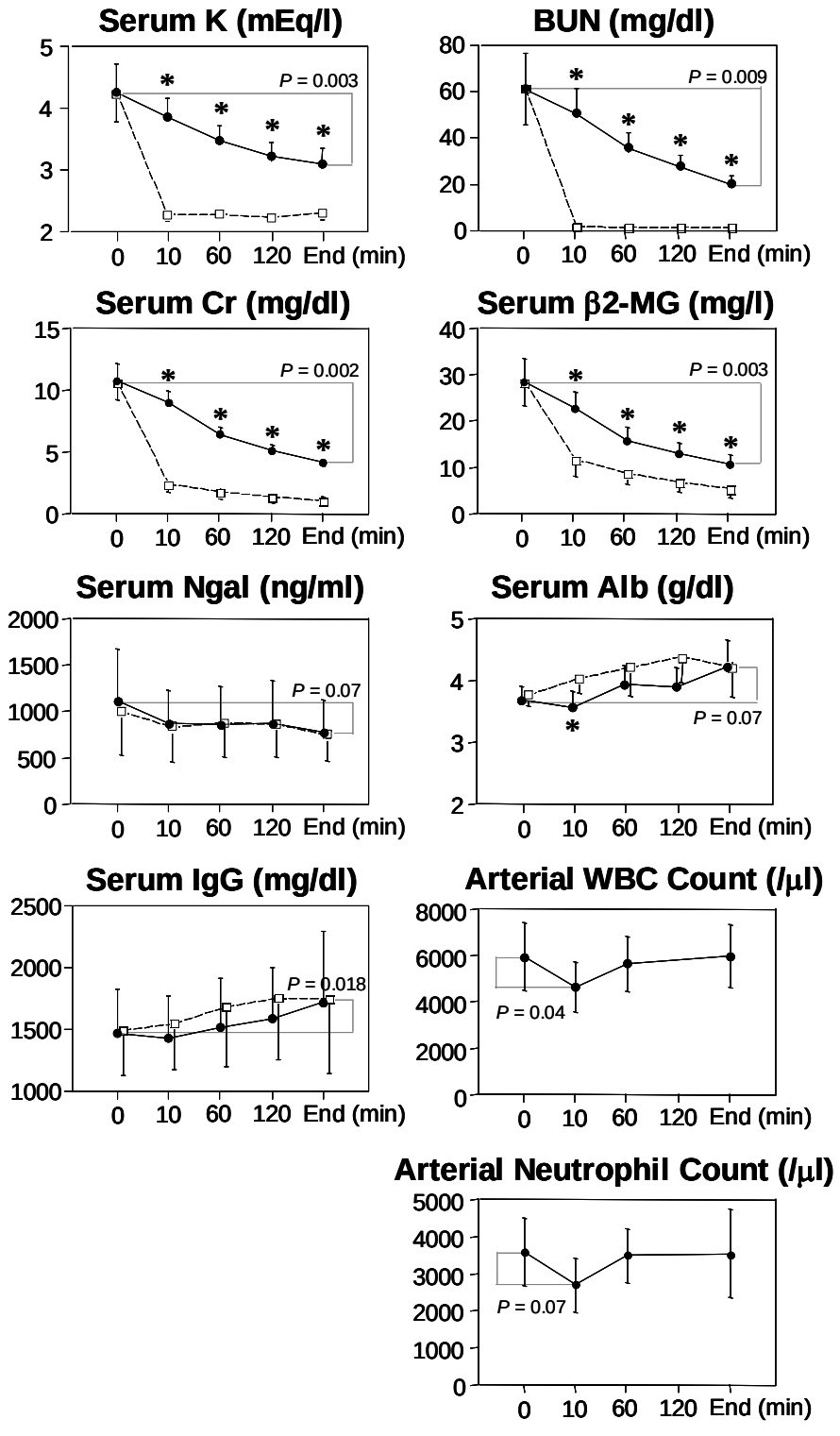

Table 1. Baseline characteristics of 79 MHD patients in two dialysis centers

Values are expressed as mean ± SD or median (25 – 75 percentile). Differences between two centers were compared (a) by Student's t test or (b) by Welch's test, assuming equal or unequal variance, respectively. MHD, maintenance hemodialysis; PTH, parathyroid hormone; CRP, C-reactive protein; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; TIBC, total iron binding capacity; UIBC, unsaturated iron binding capacity; TSAT, transferring saturation; Kt/Vsp, single-pool Kt/V; nPCR, normalized protein catabolic rate; CGR, creatinine generation rate.

Dialysis Center	M (*n* = 23)	N (*n* = 56)	Difference (*P*)
Pre-dialysis Ngal (ng/ml)	909 ± 276	954 ± 335	0.57
Post-dialysis Ngal (ng/ml)	664 ± 210	758 ± 248	0.12
Ngal Removal Ratio (%)	27 ± 11	19 ± 14	0.02[a]
Na (mEq/l)	139 ± 3	138 ± 3	0.07
Cl (mEq/l)	104 ± 3	104 ± 4	0.52
K (mEq/l)	4.9 ± 0.7	5.1 ± 0.7	0.26
Ca (mg/dl)	8.7 ± 0.8	9.0 ± 0.7	0.10
Normalized Ca (mg/dl)	8.9 ± 0.7	9.1 ± 0.7	0.15
Phosphorus (mg/dl)	4.6 ± 0.8	5.6 ± 1.5	<0.001[b]
Intact PTH (pg/ml)	115 (66 - 213)	191 (133 - 357)	<0.001[b]
BUN (mg/dl)	68 ± 14	70 ± 14	0.62
Creatinine (mg/dl)	11.3 ± 2.6	11.7 ± 2.8	0.64
UA (mg/dl)	7.5 ± 1.4	6.6 ± 1.3	0.007[a]
GOT (IU/l)	11 (7 - 17)	12 (10 - 16)	0.93
GPT (IU/l)	8 (6 - 14)	9 (8 - 13)	0.61
LDH (IU/l)	160 (142 - 184)	170 (156 - 199)	0.13
γGTP (IU/l)	20 (12 - 33)	19 (12 - 25)	0.38
ALP (IU/l)	189 (155 - 224)	263 (201 - 323)	0.04[a]
CPK (IU/l)	88 (58 - 113)	79 (55 - 120)	0.68
Total Protein (g/dl)	6.6 ± 0.4	6.4 ± 0.4	0.01[a]
Albumin (g/dl)	3.9 ± 0.3	3.9 ± 0.3	0.43
Choline Esterase (IU/l)	190 ± 43	233 ± 66	0.006[a]
Total Cholesterol (mg/dl)	158 ± 39	155 ± 35	0.76
Triglyceride (mg/dl)	86 (63 - 152)	81 (63 - 126)	0.47
CRP (mg/dl)	0.10 (0.00 - 0.20)	0.07 (0.05 - 0.12)	0.35
pH	7.2 ± 0.8	7.3 ± 0.0	0.53
pCO_2 (mmHg)	37 ± 3	35 ± 4	0.04[a]
pO_2 (mmHg)	106 ± 17	106 ± 20	0.82
HCO_3^- (mEq/l)	22 ± 1	18 ± 2	<0.001[b]
Base Excess (mEq/l)	-2.5 (-3.3 to -1.1)	-7.0 (-9.0 to -5.6)	<0.001[a]
Anion Gap (mEq/l)	18.9 ± 1.9	21.1 ± 2.9	0.001[a]
White Blood Cell (/μl)	6032 ± 2109	5636 ± 1507	0.35
Neutrophil (/μl)	3787 ± 1402	3818 ± 1203	0.92
Red Blood Cell ($x10^6$/μl)	3.17 ± 0.46	3.50 ± 0.47	0.005[a]
Hemoglobin (g/dl)	9.5 ± 1.3	10.8 ± 1.4	<0.001[a]
Hematocrit (%)	29.3 ± 3.5	34.1 ± 4.1	<0.001[a]
MCV (fl)	93 ± 5	98 ± 5	0.001[a]
MCH (pg)	30.3 ± 2.2	30.9 ± 2.1	0.23
MCHC (%)	32.5 ± 1.3	31.6 ± 1.1	0.003[a]
Reticulocyte (per mili)	10.8 ± 4.9	12.7 ± 5.9	0.19
Platelet ($x10^3$/μl)	171 ± 55	178 ± 58	0.60
Ferritin (ng/ml)	55 (34 - 144)	165 (75 - 230)	<0.001[b]
Iron (mg/dl)	75 ± 26	65 ± 35	0.23
TIBC (mg/dl)	247 ± 41	250 ± 51	0.78
UIBC (mg/dl)	166 ± 57	185 ± 53	0.16
TSAT(Fe/TIBC, %)	31 ± 12	26 ± 12	0.10
Pre-dialysis BMI (kg/m^2)	22.7 ± 4.8	22.0 ± 3.9	0.50
Post-dialysis BMI (kg/m^2)	21.7 ± 4.5	21.0 ± 3.8	0.48
Kt/Vsp	1.4 ± 0.3	1.3 ± 0.3	0.09
nPCR (g/kg/day)	0.92 ± 0.19	0.91 ± 0.18	0.75
%CGR (%)	103 ± 28	102 ± 26	0.96
Age (year)	62 ± 14	63 ± 11	0.67
Hemodialysis Period (month)	40 (22 - 79)	87 (53 - 155)	0.002[b]

Table 2. Correlation of parameters with pre-dialysis serum Ngal levels by univariate linear regression analysis (top 25 of 50 parameters)

Among clinical parameters shown in Table 1, 25 which had relatively strong correlation coefficients of either > 0.17 or < -0.17 are shown. Top 8 strongest correlations are highlighted by underlines. The rest are shown in Supplementary Table 2.

	Center M (*n* = 23)		Center N (*n* = 56)		Centers M+N (*n* = 79)	
	r	***P***	***r***	***P***	***r***	***P***
White Blood Cell	0.45	0.03	0.69	<0.001	**0.63**	**<0.001**
Neutrophil	0.40	0.06	0.69	<0.001	**0.63**	**<0.001**
Creatinine	0.59	0.003	0.63	<0.001	**0.62**	**<0.001**
%CGR	0.54	0.008	0.60	<0.001	**0.58**	**<0.001**
Anion Gap	0.61	0.002	0.56	<0.001	**0.54**	**<0.001**
Cl	-0.53	0.009	-0.49	<0.001	**-0.50**	**<0.001**
Platelet	0.47	0.02	0.48	<0.001	**0.49**	**<0.001**
nPCR	0.44	0.04	0.37	0.006	**0.38**	**0.001**
BUN	0.39	0.07	0.35	0.009	**0.36**	**0.001**
Age	-0.20	0.35	-0.38	0.003	**-0.34**	**0.002**
Hemodialysis Period	0.21	0.34	0.37	0.005	**0.34**	**0.002**
Albumin	0.11	0.63	0.39	0.003	**0.33**	**0.003**
Ca	0.26	0.24	0.34	0.01	**0.32**	**0.004**
Kt/Vsp	0.19	0.39	0.39	0.004	**0.32**	**0.004**
Na	-0.26	0.23	-0.30	0.03	**-0.31**	**0.01**
Phosphorus	0.19	0.38	0.31	0.02	**0.28**	**0.01**
Choline Esterase	0.33	0.14	0.23	0.09	**0.26**	**0.02**
Normalized Ca	0.23	0.29	0.25	0.07	**0.25**	**0.03**
UA	0.11	0.61	0.35	0.02	**0.25**	**0.04**
Total Protein	0.15	0.50	0.28	0.04	**0.25**	**0.03**
pH	-0.22	0.32	-0.31	0.02	**-0.24**	**0.03**
Red Blood Cell	-0.15	0.51	0.32	0.02	**0.24**	**0.04**
LDH	-0.14	0.53	-0.20	0.14	**-0.18**	**0.12**
pCO2	0.11	0.63	0.20	0.13	**0.18**	**0.12**
Hemoglobin	-0.26	0.24	0.27	0.05	**0.18**	**0.11**

Table 3. Determinants of serum Ngal levels by multivariate linear regression analysis

Variable	Standardized Coefficient (β)	*T*	*P*
%CGR	0.44	5.4	<0.001
WBC	0.37	4.6	<0.001
Anion Gap	0.23	2.8	0.007

Table 4. Mutual association among clinical parameters which are closely correlated to serum Ngal level

Correlation coefficients which were ≥ 0.45 or ≤ -0.45 are highlighted by underlines ($P < 0.001$). All the rest of correlations were also statistically significant ($P \leq 0.008$).

	Neutrophil	Platelet	Cr	%CGR	Anion Gap	nPCR	Cl
WBC	0.92	0.62	0.37	0.34	0.36	0.22	-0.33
Neutrophil		0.58	0.36	0.33	0.41	0.24	-0.35
Platelet			0.30	0.37	0.33	0.25	-0.38
Cr				0.80	0.47	0.26	-0.51
%CGR					0.33	0.36	-0.45
Anion Gap						0.45	-0.45
nPCR							-0.34

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Table 5. Comparison of plasma levels of markers in aorta and renal vein

Blood was collected from aorta and renal vein during coronary angiography in 15 patients not receiving MHD (non-HD) and 15 receiving MHD (HD). Ngal, creatinine (Cr), albumin (Alb) and choline esterase (ChE) were examined. %Ngal and %Cr indicate relative values normalized for levels in aorta in each subject. Statistically significant difference between aorta and renal vein: $^{a}P = 0.003$, $^{b}P = 0.001$, $^{c}P < 0.001$.

	Ngal		%Ngal		Cr		%Cr	
	non-HD	HD	non-HD	HD	non-HD	HD	non-HD	HD
Aorta	92 ± 62	765 ± 183	100 ± 0	100 ± 0	0.90 ± 0.47	7.6 ± 1.6	100 ± 0	100 ± 0
Renal Vein	81 ± 59[a]	768 ± 213	87 ± 12[b]	100 ± 10	0.73 ± 0.39[c]	7.4 ± 1.6	81 ± 10[c]	98 ± 4

	Alb		ChE	
	non-HD	HD	non-HD	HD
Aorta	4.0 ± 0.5	3.7 ± 0.3	274 ± 69	216 ± 47
Renal Vein	4.0 ± 0.5	3.6 ± 0.3	275 ± 69	212 ± 43

Table 6. Causes of admission in 79 MHD patients during the past 2 years, and recent serum Ngal levels

Causes of admission (Adm) were categorized as cardiovascular disease (CVD), infection or others. Numbers of patients are indicated in brackets. [a]Category, [b]Number of cases, [c]Median serum Ngal, [d]25 - 75 percentile.[e]One patient had two admissions by acute myocardial infarction and by follow-up coronary angiography. [f]Admitted twice by infection. [g]Operations for hemorrhoid (2 cases), secondary hyperparathroidism and embolisation of polycystic kidney disease. [h]Biopsies of nasal polyp and thyroid that exhibited no malignancy. [i]Radical operation.

Total[a]	No Adm	History of Adm			
79[b]	46	33	by CVD	by Infection	by Other Causes
			12	7	14
943[c]	963	908	973	701	942
(701 - 1160)[d]	(729 - 1171)	(646 - 1166)	(682 - 1175)	(612 - 1213)	(696 - 1149)
			ischemic heart disease (4)[b,e]	pneumonia (2)	operation (4)[g]
			stroke (3)	pyelonephritis (1)	bone fracture (2)
			congestive heart failure (2)	catheter infection (1)	biopsy (2)[h]
			deep vein thrombosis (1)	nasopharyngitis (1)	bronchia asthma (1)
			hypotension (1)	enteritis (1)[f]	tooth removal (1)
			arrythmia (1)	cholangitis (1)	sudden deafness (1)
					acute pancreatitis (1)
					colon cancer resection (1)[i]
					laryngial polypectomy (1)

SUPPLEMENTAL DATA

Supplementary Table 1. Baseline characteristics of 16 cases that were excluded in the primary analysis, in comparison with the rest of 79 MHD patients

Values are expressed as mean ± SD or median (25 – 75 percentile). The excluded patients were three under treatment with Mycobacterium tuberculosis, one suffering from lung cancer, and 12 having CRP levels more than 0.5 mg/dl (ranging 0.53 – 4.39, *n* = 12).

Dialysis Center	Excluded (*n* = 16)	Centers M+N (*n* = 79)	Difference (*P*)
Pre-dialysis Ngal (ng/ml)	**1197 ± 486**	941 ± 318	0.009[a]
Post-dialysis Ngal (ng/ml)	**942 ± 449**	731 ± 240	0.09[b]
Ngal Removal Ratio (%)	**22 ± 17**	21 ± 14	0.87
Na (mEq/l)	**138 ± 3**	139 ± 3	0.31
Cl (mEq/l)	**103 ± 4**	104 ± 4	0.38
K (mEq/l)	**5.3 ± 0.8**	5.1 ± 0.7	0.34
Ca (mg/dl)	**8.5 ± 0.9**	8.9 ± 0.8	0.11
Normalized Ca (mg/dl)	**9.0 ± 0.8**	9.1 ± 0.7	0.68
Phosphorus (mg/dl)	**6.3 ± 2.1**	5.3 ± 1.4	0.07
Intact PTH (pg/ml)	**232 (111 - 362)**	164 (94 - 279)	0.28
BUN (mg/dl)	**74 ± 21**	69 ± 14	0.38
Creatinine (mg/dl)	**10.0 ± 2.8**	11.6 ± 2.7	0.04[a]
UA (mg/dl)	**7.1 ± 6.4**	6.9 ± 1.4	0.64
GOT (IU/l)	**13 (8 - 19)**	12 (10 - 16)	0.34
GPT (IU/l)	**9 (5 - 15)**	9 (7 - 13)	0.76
LDH (IU/l)	**180 (161 - 216)**	168 (154 - 190)	0.30
γGTP (IU/l)	**26 (13 - 41)**	19 (12 - 26)	0.24
ALP (IU/l)	**358 (196 - 412)**	245 (187 - 310)	0.14
CPK (IU/l)	**69 (34 - 116)**	79 (57 - 118)	0.32
Total Protein (g/dl)	**6.4 ± 0.5**	6.4 ± 0.4	0.88
Albumin (g/dl)	**3.6 ± 0.5**	3.9 ± 0.3	0.001[a]
Choline Esterase (IU/l)	**206 ± 58**	221 ± 63	0.39
Total Cholesterol (mg/dl)	**160 ± 30**	156 ± 36	0.69
Triglyceride (mg/dl)	**107 (67 - 163)**	81 (63 - 141)	0.89
CRP (mg/dl)	**0.70 (0.51 - 1.47)**	0.09 (0.05 - 0.14)	0.003[b]
pH	**7.3 ± 0.1**	7.3 ± 0.4	0.81
pCO_2 (mmHg)	**33 ± 5**	35 ± 4	0.11
pO_2 (mmHg)	**95 ± 18**	106 ± 19	0.028[a]
HCO_3^- (mEq/l)	**17 ± 3**	19 ± 3	0.016[a]
Base Excess (mEq/l)	**-8.4 (-10.3 to -5.1)**	-5.8 (-7.8 to -3.3)	0.029[a]
Anion Gap (mEq/l)	**22.7 ± 3.5**	20.4 ± 2.8	0.007[a]
White Blood Cell (/μl)	**6342 ± 1991**	5751 ± 1699	0.22
Neutrophil (/μl)	**4357 ± 2027**	3808 ± 1260	0.34
Red Blood Cell ($x10^6$/μl)	**3.34 ± 0.44**	3.40 ± 0.48	0.61
Hemoglobin (g/dl)	**10.3 ± 1.7**	10.4 ± 1.5	0.75
Hematocrit (%)	**32.6 ± 4.4**	32.7 ± 4.5	0.93
MCV (fl)	**98 ± 8**	96 ± 6	0.48
MCH (pg)	**30.9 ± 2.9**	30.8 ± 2.1	0.89
MCHC (%)	**31.5 ± 0.9**	31.9 ± 1.2	0.21
Reticulocyte (per mili)	**14.5 ± 7.4**	12.1 ± 5.6	0.15
Platelet ($x10^3$/μl)	**179 ± 60**	176 ± 57	0.86
Ferritin (ng/ml)	**194 (101 - 468)**	144 (52 - 199)	0.08
Iron (mg/dl)	**57 ± 27**	68 ± 33	0.22
TIBC (mg/dl)	**215 ± 50**	249 ± 48	0.01[a]
UIBC (mg/dl)	**158 ± 63**	180 ± 55	0.17
TSAT(Fe/TIBC, %)	**29 ± 17**	28 ± 12	0.84
Pre-dialysis BMI (kg/m^2)	**21.1 ± 4.8**	22.2 ± 4.2	0.37
Post-dialysis BMI (kg/m^2)	**19.9 ± 4.8**	21.2 ± 4.1	0.28
Kt/Vsp	**1.5 ± 0.2**	1.3 ± 0.3	0.04[a]
nPCR (g/kg/day)	**1.01 ± 0.24**	0.91 ± 0.18	0.07
%CGR (%)	**97 ± 27**	102 ± 27	0.47
Age (year)	**67 ± 8**	63 ± 12	0.19
Hemodialysis Period (month)	**106 ± 73**	73 (39 - 141)	0.47

Supplementary Table 2. Correlation of parameters with pre-dialysis serum Ngal levels by univariate linear regression analysis (all 50 parameters)

	Center M (*n* = 23)		Center N (*n* = 56)		Centers M+N (*n* = 79)	
	r	***P***	***r***	***P***	***r***	***P***
White Blood Cell	0.45	0.03	0.69	<0.001	**0.63**	**<0.001**
Neutrophil	0.40	0.06	0.69	<0.001	**0.63**	**<0.001**
Creatinine	0.59	0.003	0.63	<0.001	**0.62**	**<0.001**
%CGR	0.54	0.008	0.60	<0.001	**0.58**	**<0.001**
Anion Gap	0.61	0.002	0.56	<0.001	**0.54**	**<0.001**
Cl	-0.53	0.009	-0.49	<0.001	**-0.50**	**<0.001**
Platelet	0.47	0.02	0.48	<0.001	**0.49**	**<0.001**
nPCR	0.44	0.04	0.37	0.006	**0.38**	**0.001**
BUN	0.39	0.07	0.35	0.009	**0.36**	**0.001**
Age	-0.20	0.35	-0.38	0.003	**-0.34**	**0.002**
Hemodialysis Period	0.21	0.34	0.37	0.005	**0.34**	**0.002**
Albumin	0.11	0.63	0.39	0.003	**0.33**	**0.003**
Ca	0.26	0.24	0.34	0.01	**0.32**	**0.004**
Kt/Vsp	0.19	0.39	0.39	0.004	**0.32**	**0.004**
Na	-0.26	0.23	-0.30	0.03	**-0.31**	**0.01**
Phosphorus	0.19	0.38	0.31	0.02	**0.28**	**0.01**
Choline Esterase	0.33	0.14	0.23	0.09	**0.26**	**0.02**
Normalized Ca	0.23	0.29	0.25	0.07	**0.25**	**0.03**
UA	0.11	0.61	0.35	0.02	**0.25**	**0.04**
Total Protein	0.15	0.50	0.28	0.04	**0.25**	**0.03**
pH	-0.22	0.32	-0.31	0.02	**-0.24**	**0.03**
Red Blood Cell	-0.15	0.51	0.32	0.02	**0.24**	**0.04**
LDH	-0.14	0.53	-0.20	0.14	**-0.18**	**0.12**
pCO2	0.11	0.63	0.20	0.13	**0.18**	**0.12**
Hemoglobin	-0.26	0.24	0.27	0.05	**0.18**	**0.11**
MCH	-0.11	0.61	-0.23	0.09	**-0.17**	**0.13**
Hematocrit	-0.18	0.41	0.23	0.09	**0.17**	**0.13**
MCV	-0.23	0.29	-0.23	0.09	**-0.16**	**0.15**
Total Cholesterol	0.31	0.15	0.09	0.52	**0.16**	**0.17**
Triglyceride	0.19	0.38	0.11	0.60	**0.14**	**0.34**
Base Excess	-0.28	0.21	-0.12	0.37	**-0.13**	**0.26**
GOT	-0.08	0.72	-0.16	0.24	**-0.12**	**0.29**
Ferritin	0.33	0.13	0.08	0.54	**0.12**	**0.28**
UIBC	-0.09	0.68	0.18	0.20	**0.12**	**0.31**
CRP	0.05	0.84	0.15	0.27	**0.11**	**0.33**
pO2	0.02	0.92	0.14	0.31	**0.11**	**0.35**
TIBC	-0.23	0.34	0.18	0.19	**0.10**	**0.37**
Reticulocyte	0.36	0.10	-0.01	0.94	**0.10**	**0.36**
GPT	-0.31	0.16	0.03	0.81	**-0.08**	**0.49**
HCO3-	-0.17	0.45	-0.03	0.81	**-0.08**	**0.51**
K	0.14	0.51	0.04	0.78	**0.08**	**0.50**
MCHC	0.10	0.64	0.12	0.39	**0.08**	**0.50**
Intact PTH	-0.21	0.34	0.11	0.44	**0.07**	**0.57**
CPK	-0.12	0.59	0.13	0.33	**0.07**	**0.57**
TSAT	-0.10	0.64	-0.03	0.82	**-0.06**	**0.60**
gGTP	0.05	0.84	0.09	0.53	**0.05**	**0.66**
Iron	-0.24	0.28	0.01	0.94	**-0.04**	**0.73**
ALP	-0.11	0.61	0.06	0.69	**0.04**	**0.70**
Pre-dialysis BMI	0.29	0.18	-0.10	0.47	**-0.01**	**0.95**
Post-dialysis BMI	0.31	0.15	-0.10	0.50	**-0.01**	**0.97**

Supplementary Table 3. Multivariate linear regression analysis of parameters determining serum Ngal levels in 95 MHD patients without excluded cases

Variable	Standardized Coefficient (β)	*T*	*P*
WBC	0.34	4.6	<0.001
CRP	0.32	4.3	<0.001
%CGR	0.31	4.1	<0.001
Anion Gap	0.25	3.2	0.002