North Clin Istanb 2017;4(1):36–42
doi: 10.14744/nci.2017.59002

Original Article NEPHROLOGY

Neutrophil gelatinase-associated lipocalin reflects the severity of anemia without iron deficiency and secondary hyperparathyroidism in hemodialysis patients

Irem Pembegul Yigit,[1] Ramazan Ulu,[2] Nevzat Gozel,[3] Hulya Taskapan,[4] Necip Ilhan,[5] Ayhan Dogukan[2]

[1]Department of Nephrology, Malatya Government Hospital, Malatya, Turkey
[2]Department of Nephrology, Firat University Faculty of Medicine, Elazig, Turkey
[3]Department of Internal Medicine, Firat University Faculty of Medicine, Elazig, Turkey
[4]Department of Nephrology, Inonu University Faculty of Medicine, Malatya, Turkey
[5]Department of Biochemistry, Firat University Faculty of Medicine, Elazig, Turkey

ABSTRACT

OBJECTIVE: Secondary hyperparathyroidism (SHPT) and anemia are the primary and most common complications in patients receiving hemodialysis (HD). Neutrophil gelatinase-associated lipocalin (NGAL) is a new marker to assess iron deficiency and manage iron therapy for HD patients. The aim of this study was to determine any association between serum NGAL level and anemia without iron deficiency in patients with SHPT on chronic HD.

METHODS: Total of 61 SHPT patients on chronic HD were enrolled in the study and divided into 3 groups: mild SHPT group (n=17), moderate SHPT group (n=21), and severe SHPT group (n=23). Hemogram, biochemical assays, and level of ferritin, high sensitivity C-reactive protein (hs-CRP), and NGAL were evaluated in all groups.

RESULTS: Serum NGAL level was significantly higher and hemoglobin (Hb) level was significantly lower in severe SHPT patients compared with both mild and moderate SHPT patients. Furthermore, in severe SHPT group, serum NGAL level was significantly positively correlated with serum parathyroid hormone (r=0.79; p=0.00) and hs-CRP (r=0.52; p=0.01) level and negatively correlated with serum Hb (r=-0.56; p=0.00) level.

CONCLUSION: SHPT was important factor affecting anemia in HD patients. Even when iron deficiency anemia is excluded in patients with SHPT, there was significant negative correlation between serum NGAL and Hb.

Keywords: Anemia; hemodialysis; hyperparathyroidism; neutrophil gelatinase-associated lipocalin.

Received: November 11, 2016 *Accepted:* January 23, 2017 *Online:* May 10, 2017

Correspondence: Dr. Irem Pembegul YIGIT. Malatya Devlet Hastanesi, Nefroloji Klinigi, Malatya, Turkey.
Tel: +90 422 - 211 20 48 e-mail: pembegulmd@yahoo.com

Chronic kidney disease (CKD) is defined as presence of kidney damage or decreased kidney function (estimated glomerular filtration rate <60 mL/min/1.73 m^2) for 3 or more months, irrespective of cause. Prevalence and incidence of CKD continues to increase globally. Secondary hyperparathyroidism (SHPT) and anemia are the principal and most commonly seen complications in patients with CKD, and especially affect patients receiving hemodialysis (HD) [1]. Causes of SHPT include phosphate retention, decreased free calcium concentration, decreased 1,25-dihydroxyvitamin D (calcitriol) concentration, and increased fibroblast growth factor 23 concentrations, with reduced expression of vitamin D receptors, calcium-sensing receptors, fibroblast growth factor receptors, and klotho in the parathyroid glands. Most observational studies have reported associations between elevated parathyroid hormone (PTH) level and mortality [2–4]. Kidney Disease: Improving Global Outcomes guidelines suggest maintaining serum PTH value in HD patients in the range of 2 to 9 times upper reference limit for the assay due to technical differences in the laboratory [5].

Mechanism underlying anemia in patients on maintenance HD is attributed to several factors, including decreased erythropoietin (EPO) production, hyporesponsiveness to EPO, low iron stores, multiple instances of blood sampling, gastrointestinal bleeding, dietary restrictions, decreased intestinal absorption due to phosphate medication, dialysis inadequacy, SHPT, and chronic inflammation [6–8]. Among these, EPO deficiency is considered the most important cause. PTH acts directly or indirectly to increase intracellular calcium concentrations, and this leads to critical conclusions, including anemia, immune dysfunction, neurotoxicity, and impairment of vascular reactivity. At the same time, HD patients with SHPT suffer from excessive oxidative stress and inflammation [9, 10].

Neutrophil gelatinase-associated lipocalin (NGAL) is small 25 kDa glycoprotein, a member of lipocalin superfamily that is rapidly released from cells such as renal tubules, liver hepatocytes, endothelial, and smooth muscle cells in response to inflammation and ischemia [11, 12]. NGAL, identified as component of neutrophil granules, inhibits bacterial growth by depleting their intracellular iron stores. NGAL has also been shown to induce apoptosis of primary bone marrow cells, including erythroid progenitor cells, and to inhibit erythroid cell production, leading to anemia [13, 14]. Previous study demonstrated relationship between iron deficiency anemia and serum NGAL level in chronic HD patients [15]. The aim of present study was to test hypothesis whether NGAL might be correlated with severity of SHPT and anemia without iron deficiency.

MATERIALS AND METHODS

Patients

Study was conducted with 61 chronic HD patients (mean age: 52.21±11.6 years; male/female: 29/32). All patients were anuric and were undergoing standard bicarbonate dialysis using standard biocompatible HD membrane 3 times a week for about 4 hours each session in the hemodialysis unit of Firat University. Blood flow rates were 250 to 300 mL/min and dialysate flow rates were 500 mL/min. Ultrafiltration varied according to the patients' actual weight. None had received intravenous iron or red cell blood transfusion in previous 2 months. Exclusion criteria of the study were as follows: patients who had been receiving HD for less than 6 months; age <18 or >70 years; serum PTH level <150 pg/mL; presence of malignancy; presence or recent history of bleeding; B_{12} or folic-acid deficiency; iron deficiency anemia (transferring saturation value <20% and/or serum ferritin level <200 ng/mL); hemoglobinopathies; hemolytic anemias; liver, thyroid, or infectious disease; treatment with immunosuppressive drugs; and unwillingness to participate in the study. Causes of renal failure among HD patients were diabetic nephropathy in 25 (41%) patients, hypertension in 20 (32.8%), chronic glomerulonephritis in 4 (6.6%), polycystic kidney disease in 3 (4.9%), other cause in 4 (6.6%), and unknown cause in 5 (8.1%) patients. Mean duration of HD was 30.36±15.70 months (range: 6–68 months). Only 3 patients had permanent central venous catheters and the remainder (n=58) had arteriovenous

fistula. Although there is no precise cut-off level determining risk groups, as shown in the Dialysis Outcomes and Practice Patterns Study [10], patients with PTH over 600 have high risk of mortality; therefore, patients were divided into 3 groups based on PTH level: Group 1=150<PTH≤300 pg/mL, Group 2=301≤PTH≤600 pg/mL, and Group 3=601pg/mL≤PTH. The study was approved by the Local ethics committee and all patients provided written informed consent. All participants underwent detailed clinical examination. Demographic information and medical history of patients were obtained at baseline by interview with patients and review of medical records.

Blood sampling and assay methods

Blood samples were taken after an overnight fast at between 8:00 and 9:00 am before HD session. At the end of HD session, blood pump speed was reduced to <80 mL/min and blood samples was obtained at 2 minutes post dialysis from arterial dialysis tubing for calculation of adequacy of dialysis using $(K_{urea} \times Td) / V_{urea}$ for urea measurement. Laboratory analysis, including creatinin, serum iron (SI), total iron binding capacity (TIBC), albumin, calcium, phosphorus, alkaline phosphates (ALP), PTH, and hemoglobin (Hb) levels, was conducted using standard laboratory methods in central laboratory on the same day. For blood samples, complete blood count was assessed with Siemens Advia 2120i System (Siemens Healthineers, Erlangen, Germany), and biochemical parameters with Olympus AU 2700 autoanalyzer (Olympus Corp., Tokyo, Japan) using specific kits for devices. Ferritin level was determined with Siemens branded-eligible kits (Siemens Healthineers, Erlangen, Germany). PTH level was analyzed with Siemens Advia Centaur XP Immunoassay System (Siemens Healthineers, Erlangen, Germany) using specific kits. Serum and plasma samples were stored at -20°C until measurement of NGAL and high sensitivity-C-reactive protein (hs-CRP). NGAL (Catalog No: EK0853) was examined using enzyme-linked immunosorbent assay method (Boster Biological Technology Co., Pleasanton, CA, USA; NGAL sensitivity 510 pg/mL). hs-CRP level was measured with Immulite 2000 instruments using chemiluminescent method (detection range: 0.1–250 mg/L; intra assay precision: coefficient of variation [CV]% <10; inter assay precision: CV% <10) (Siemens Healthineers, Erlangen, Germany).

Transferrin saturation was calculated using following formula: SI/TIBC x 100.

Kt/V calculation was made online by submitting patient dialysis technique and test results to Hypertension Dialysis and Clinical Nephrology website (www.hdcn.com).

Statistical analysis

Statistical analyses were performed using SPSS for Windows, version 16.0 (SPSS Inc., Chicago, IL, USA). Descriptive analyses were presented as mean±SD. Normality of variables was assessed using Kolmogorov-Smirnov test. Kruskal-Wallis test was conducted to compare parameters between groups, and Mann-Whitney U-test was performed to test significance of pairwise differences for multiple comparisons. Pearson correlation analysis was employed to test correlations between NGAL and other variables considered in the study. Linear regression analysis employed Pearson coefficients as appropriate. Multiple regression analysis was used to determine independent factors affecting dependent variable (NGAL). Data were expressed as partial correlation coefficients (β) and p value. The results were considered significant if p value was <0.05.

RESULTS

All clinical and biochemical data are presented in Table 1.

There was no significant difference between groups with respect to age, gender, HD duration, Kt/V, neutrophil count, or body mass index. NGAL level was significantly higher in severe SHPT group ($p<0.05$). Hb level was significantly lower in patients with severe SHPT compared with both mild and moderate SHPT patients. Although serum ferritin, calcium-phosphorus (CaxP) product, and ALP levels were higher in patients with

TABLE 1. Clinical and biochemical data of patients on hemodialysis

	Group 1 (mild) 150<PTH≤300	Group 2 (moderate) 301≤PTH≤600	Group 3 (severe) 601≤PTH	p
Age (years)	52.21±11.60	51.33±13.53	52.60±10.01	NS
Gender (F/M)	10/7	9/12	13/10	NS
DM (%)	8 (47)	8 (38)	9 (39.11)	NS
HD duration (months)	28.70±20.87	27.66±16.06	29.01±14.23	NS
Kt/V	1.35±0.07	1.34±0.08	1.35±0.08	NS
BMI (kg/m^2)	21.58±2.69	22.73±2.46	22.62±2.58	NS
Neutrophil count (per mm^3)	6348.26±1174.12	6819.35±1326.12	7129.42±1425.08	NS
Hb (g/dL)	11.71±0.23	11.13±0.34	10.50±0.50	a,b,c
SI (µd/dL)	51.15±12.25	54.48±11.09	55.34±12.29	NS
Transferrin saturation (%)	34.94±8.25	32.23±3.91	36.38±10.06	NS
Ferritin (ng/mL)	275.76±57.67	299.19±77.46	305.43±75.19	NS
Ca (mg/dL)	8.73±0.61	8.96±0.78	9.02±1.12	NS
P (mg/dL)	4.28±0.76	4.31±0.69	5.12±0.84	C
CaxP (mg^2/dL^2)	42.19±8.78	46.91±9.02	50.79±14.97	NS
Albumin (g/dL)	3.89±0.48	3.64±0.39	2.88±0.62	b,c
ALP (IU/L)	103.58±25.11	97.86±31.06	120.91±50.27	NS
PTH (pg/mL)	224.94±45.38	439.52±85.72	901.73±146.98	a,b,c
hs-CRP (ng/mL)	4620.65±1125.49	5364.24±1866.82	6763.48±1103.78	b,c
NGAL (pg/mL)	2870.29±464.72	3926.24±849.06	7995.09±771.34	a,b,c

ALP: Alkaline phosphatase; BMI: Body mass index; Ca: Calcium; CaxP: Calcium x phosphorus; DM: Diabetes mellitus; F: Female; Kt/V: $K_{urea} \times Td/V_{urea}$; M: Male; Hb: Hemoglobin; HD: Hemodialysis; hs-CRP: High-sensitivity C-reactive protein; NGAL: Neutrophil gelatinase-associated lipocalin; NS: Not significant; PTH: Parathyroid hormone; SI: Serum iron. Significant difference (p<0.05) between groups; a=Group 1:2; b=Group 2:3; c=Group 1:3.

severe SHPT, there was no significant difference between these groups. No significant difference was detected between mild and moderate SHPT groups in hs-CRP (p>0.05). Medical treatments of patients are presented in Table 2. There was no relationship between vitamin D treatment, NGAL, and Hb levels.

Serum NGAL level was significantly positively correlated with serum PTH (r=0.79; p=0.00) and hs-CRP (r=0.52; p=0.01) levels, and negatively correlated with serum Hb (r=-0.56; p=0.00) level in all HD patients. All variables found to be significantly related to NGAL in univariate analysis were introduced in multivariate model using NGAL as dependent variable. After adjustment for other factors, significance was maintained for correlation between NGAL and serum Hb (β=-0.14; p=0.02) (Figure 1) and PTH (β=0.82; p=0.00) (Figure 2).

DISCUSSION

PTH is uremic marker, and patients with SHPT may have higher prevalence of serum inflammatory cytokines and oxidative stress, which are associated with high all-cause and cardiovascular-specific mortality [9, 10]. Additionally, PTH has been identified as a possible factor in development of acquired immune dysfunction. Increases in intracellular calcium levels potentially lead to increase in cellular adenylate cyclase activity; this has been suggested as mechanism by which PTH influences neutrophils via impaired migration, reduced phagocytic activity, and inhibited granulocyte chemotaxis [16, 17]. In our study, neutrophil count was not associated with severity of SHPT.

Both serum NGAL and hs-CRP levels were higher in severe SHPT group than mild or moder-

Table 2. Medical treatments of patients

	Group 1		Group 2		Group 3		p
	n	%	n	%	n	%	
Phosphate binders							
Calcium-based	10	58.82	11	52.38	11	47.82	NS
Non-calcium-based	3	17.65	8	38.09	12	52.17	a,b,c
Vitamin D, oral or intravenous	2	11.76	12	57.14	11	47.82	a,c
Paricalcitol	–	–	5	23.81	–	–	NA
Cinacalcet	–	–	–	–	3	13.04	NA

NA: Not applicable NS: Not significant. Significant difference ($p<0.05$) between groups; a=Group 1:2; b=Group 2:3; c=Group 1:3.

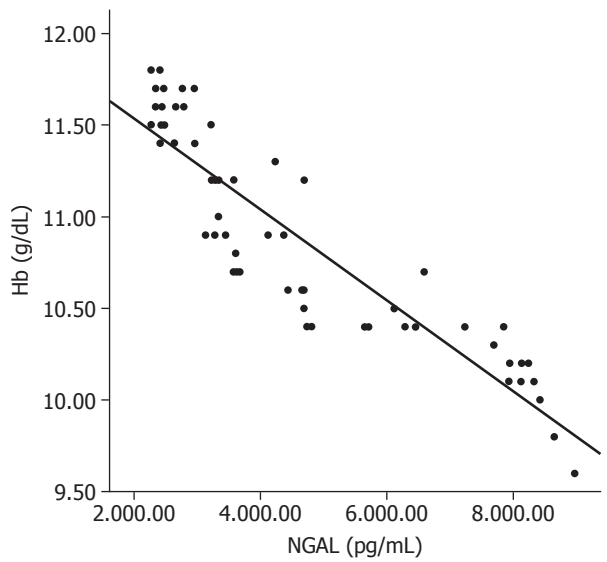

Figure 1. Correlation between serum NGAL and Hb levels.

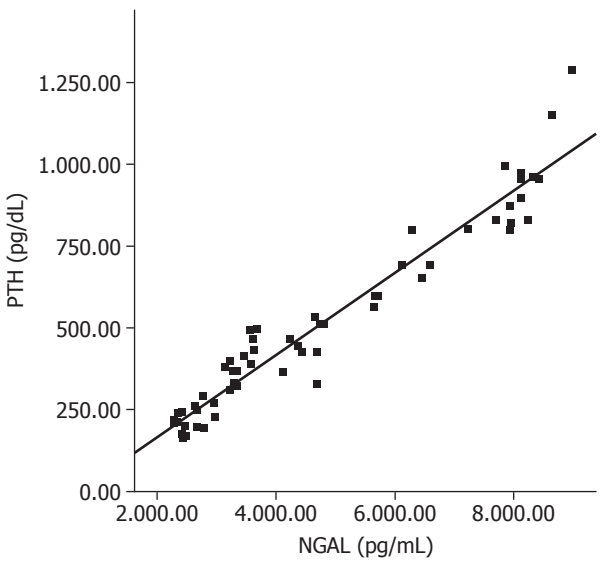

Figure 2. Correlation between serum NGAL and PTH levels.

ate SHPT groups in our study, a result that represents increased inflammation in severe SHPT patients. Additionally, correlation between NGAL and hs-CRP observed only in severe SHPT group suggests that inflammation is exacerbated with increase in PTH level. Furthermore, in a previous study, we reported that there was correlation between vascular access type and NGAL [18]. In the present study, only 3 patients had central venous permanent catheters, so we could comment on relationship between vascular access type and NGAL.

Anemia can lead to decreased quality of life (impairment of cardiac function, cognition, and exercise capacity) and increased mortality in HD patients. SHPT has been recognized as a cause of worsening anemia in normochromic and normocytic pattern. In the current study, we observed inverse association between PTH level and serum Hb concentration. This inverse relationship may be explained by elevated PTH level, which may cause resistance to EPO by increasing bone marrow fibrosis, increase osmotic fragility of red blood cells, leading to short-

ened lifespan, or inhibit proliferation of erythroid precursors. Several studies have shown important relationships between refractory anemia and higher PTH level in HD patients, as well as improvement in anemia after parathyroidectomy or calcitriol treatment [19–22].

NGAL has recently emerged as an important factor in iron homeostasis and erythrocyte growth regulation that may contribute to anemia when chronically elevated [23]. Also, previous studies have shown that NGAL levels was higher in HD patients compared with healthy subjects, and high level closely reflected iron status and systemic inflammation in HD patients [15, 24, 25].

In another study, NGAL level was measured in blood and liver after experimental induction of different types of anemia, such as blood loss and hemolytic anemia. This study showed that NGAL upregulation is not exactly mediated only by increased iron demand occurring in anemia. These results led to hypothesis that increased NGAL production may be defense mechanism against general induced hypoxia and that increased synthesis of NGAL is independent of iron status [26]. Our study demonstrated that the patients with severe SHPT but without iron deficiency anemia had highest NGAL and lowest Hb levels. Correlation analyses indicated that NGAL positively correlated with PTH level and negatively with Hb level in both severe and moderate SHPT groups. These findings suggested that in case of SHPT, NGAL may correlate with anemia, regardless of iron reserve. Correlation of NGAL with hs-CRP was observed only in severe SHPT group. As we did not include any other inflammation marker in our study, we cannot comment on contribution of inflammation to anemia observed in hyperparathyroid state, and with relatively small sample size of patients, and lack of bone marrow biopsies, constitutes important limitation in this cross-sectional study.

In conclusion, SHPT may contribute to severity of anemia in HD patients. We concluded that even when iron deficiency anemia is excluded in patients with SHPT, there was significant negative correlation between serum NGAL and Hb.

Conflict of Interest: None declared.

Financial Disclosure: The authors declared that this study has received no financial support.

Authorship contributions: Concept – I.P.Y.; Design – I.P.Y.; Supervision – H.T., A.D.; Materials – I.P.Y.; Data collection &/or processing – I.P.Y., R.U., N.G.; Analysis and/or interpretation – I.P.Y., N.I.; Literature search – I.P.Y.; Writing – I.P.Y.; Critical review – H.T., A.D.

REFERENCES

1. KDOQI; National Kidney Foundation. KDOQI Clinical Practice Guidelines and Clinical Practice Recommendations for Anemia in Chronic Kidney Disease. Am J Kidney Dis 2006;47(5 Suppl 3):S11–145.
2. Cunningham J, Locatelli F, Rodriguez M. Secondary hyperparathyroidism: pathogenesis, disease progression, and therapeutic options. Clin J Am Soc Nephrol 2011;6:913–21.
3. Block GA, Klassen PS, Lazarus JM, Ofsthun N, Lowrie EG, Chertow GM. Mineral metabolism, mortality, and morbidity in maintenance hemodialysis. J Am Soc Nephrol 2004;15:2208–18.
4. Palmer SC, Hayen A, Macaskill P, Pellegrini F, Craig JC, Elder GJ, et al. Serum levels of phosphorus, parathyroid hormone, and calcium and risks of death and cardiovascular disease in individuals with chronic kidney disease: a systematic review and meta-analysis. JAMA 2011;305:1119–27.
5. Kidney Disease: Improving Global Outcomes (KDIGO) CKD-MBD Work Group. KDIGO clinical practice guideline for the diagnosis, evaluation, prevention, and treatment of Chronic Kidney Disease-Mineral and Bone Disorder (CKD-MBD). Kidney Int Suppl. 2009;S1–130.
6. Babitt JL, Lin HY. Mechanisms of anemia in CKD. J Am Soc Nephrol 2012;23:1631–4.
7. Massry SG. Pathogenesis of the anemia of uremia: role of secondary hyperparathyroidism. Kidney Int Suppl 1983;16:S204–7.
8. Nurko S. Anemia in chronic kidney disease: causes, diagnosis, treatment. Cleve Clin J Med 2006;73:289–97.
9. Lu KC, Tseng CF, Wu CC, Yeung LK, Chen JS, Chao TY, et al. Effects of calcitriol on type 5b tartrate-resistant acid phosphatase and interleukin-6 in secondary hyperparathyroidism. Blood Purif 2006;24:423–30.
10. Tentori F, Blayney MJ, Albert JM, Gillespie BW, Kerr PG, Bommer J, et al. Mortality risk for dialysis patients with different levels of serum calcium, phosphorus, and PTH: the Dialysis Outcomes and Practice Patterns Study (DOPPS). Am J Kidney Dis 2008;52:519–30.
11. Xu S, Venge P. Lipocalins as biochemical markers of disease. Biochim Biophys Acta 2000;1482:298–307.
12. 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.

13. Goetz DH, Holmes MA, Borregaard N, Bluhm ME, Raymond KN, Strong RK. The neutrophil lipocalin NGAL is a bacteriostatic agent that interferes with siderophore-mediated iron acquisition. Mol Cell 2002;10:1033–43.
14. Miharada K, Hiroyama T, Sudo K, Danjo I, Nagasawa T, Nakamura Y. Lipocalin 2-mediated growth suppression is evident in human erythroid and monocyte/macrophage lineage cells. J Cell Physiol 2008;215:526–37.
15. Bolignano D, Coppolino G, Romeo A, De Paola L, Buemi A, Lacquaniti A, et al. Neutrophil gelatinase-associated lipocalin (NGAL) reflects iron status in haemodialysis patients. Nephrol Dial Transplant 2009;24:3398–403.
16. Geara AS, Castellanos MR, Bassil C, Schuller-Levis G, Park E, Smith M, et al. Effects of parathyroid hormone on immune function. Clin Dev Immunol 2010;2010. pii:418695.
17. Shurtz-Swirski R, Shkolnik T, Shasha SM. Parathyroid hormone and the cellular immune system. Nephron 1995;70:21–4.
18. Yigit IP, Celiker H, Dogukan A, Ilhan N, Gurel A, Ulu R, et al. Can serum NGAL levels be used as an inflammation marker on hemodialysis patients with permanent catheter? Ren Fail 2015;37:77–82.
19. Lin CL, Hung CC, Yang CT, Huang CC. Improved anemia and reduced erythropoietin need by medical or surgical intervention of secondary hyperparathyroidism in hemodialysis patients. Ren Fail 2004;26:289–95.
20. Chen C, Wu H, Zhong L, Wang X, Xing ZJ, Gao BH. Impacts of parathyroidectomy on renal anemia and nutritional status of hemodialysis patients with secondary hyperparathyroidism. Int J Clin Exp Med 2015;8:9830–8.
21. Goicoechea M, Vazquez MI, Ruiz MA, Gomez-Campdera F, Perez-García R, Valderrábano F. Intravenous calcitriol improves anaemia and reduces the need for erythropoietin in haemodialysis patients. Nephron 1998;78:23–7.
22. Neves PL, Triviño J, Casaubon F, Santos V, Mendes P, Romão P, et al. Elderly patients on chronic hemodialysis with hyperparathyroidism: increase of hemoglobin level after intravenous calcitriol. Int Urol Nephrol 2006;38:175–7.
23. Yang J, Goetz D, Li JY, Wang W, Mori K, Setlik D, et al. An iron delivery pathway mediated by a lipocalin. Mol Cell 2002;10:1045–56.
24. Bolignano D, Lacquaniti A, Coppolino G, Donato V, Campo S, Fazio MR, et al. Neutrophil gelatinase-associated lipocalin (NGAL) and progression of chronic kidney disease. Clin J Am Soc Nephrol 2009;4:337–4.
25. Bolignano D, Coppolino G, Lombardi L, Buemi M. NGAL: a new missing link between inflammation and uremic anemia? Ren Fail 2009;31:622–3.
26. Jiang W, Constante M, Santos MM. Anemia upregulates lipocalin 2 in the liver and serum. Blood Cells Mol Dis 2008;41:169–74.