

## NEUTROPHIL GELATINASE-ASSOCIATED LIPOCALIN (NGAL) IN RELATION TO RENAL ANEMIA OF HEMODIALYSIS PATIENTS

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### Abstract

Iron status in anemic chronic HD patients was primarily assessed by serum ferritin and Transferrin saturation (TSAT). Recent evaluation revealed that serum ferritin is a less reliable index of iron storage in HD patients. NGAL has been found to have a role in iron metabolism and is an early marker for AKI. Its level was prognostic to predict initiation of renal replacement therapy. The present study is a trial to evaluate NGAL as a biomarker of renal anemia in chronic HD patients. It is conducted on 40 HD patients and 20 healthy control subjects. They are divided into 2 groups: Group 1 (healthy control group) with Hb > 13g/dL and Group 2 (40 ESRD patients on regular HD thrice weekly). Group 2 is further subdivided into group 2A (20 cases with Hb < 11 g/dl) and group 2B (20 cases with Hb < 13 g/dl and ≥ 11 g/dl). Blood samples are collected from all these individuals to measure Hb, total serum iron, serum ferritin, TSAT%, CRP and NGAL. Compared to the control group, the results of the ESRD patients on HD (group 2A and 2B) show significantly lower serum iron levels ( $p < 0.001$ ), higher serum ferritin ( $p < 0.05$  and  $p < 0.001$ , respectively), lower TSAT% ( $p < 0.001$  and  $p > 0.05$ , respectively), higher CRP ( $p < 0.001$ ) and higher serum NGAL ( $p < 0.001$  and  $p < 0.01$ , respectively). Correlation studies show: 1) Significantly strong direct correlation between serum CRP and ferritin ( $r = 0.637$ ,  $p < 0.001$ ); 2) Significantly strong inverse correlation ( $p < 0.001$ ) between NGAL and Hb ( $r = -0.631$ ), between NGAL and iron ( $r = -0.578$ ), and between NGAL and TSAT% ( $r = -0.446$ ); 3) No significant correlation between NGAL and ferritin ( $r = 0.244$ ,  $p > 0.05$ ). In conclusion, NGAL may be useful as a potential biomarker in assessment of renal anemia among HD patients.

### Keywords:

NGAL-Renal anemia-Hemodialysis.

### Introduction

Anemia secondary to chronic kidney disease (CKD) is frequently observed among chronic hemodialysis (HD) patients and constitutes an important cause of morbidity and mortality<sup>(1)</sup>.

Serum ferritin and the transferrin saturation (TSAT) were considered as primary tools in the assessment of iron status in nephropathic subjects<sup>(2)</sup>. However, recent re-evaluation of the role of serum ferritin as a reliable index of iron storage in HD patients found that it is markedly influenced by malnutrition and has important gender differences, thus making it a less than ideal tool for identifying iron deficiency<sup>(3)</sup>. So, we should look for another sensitive biomarker to evaluate iron status.

Neutrophil gelatinase-associated lipocalin (NGAL), has been found to have a role in iron metabolism by virtue of its binding with siderophores. Since serum creatinine is known to be an inadequate and late marker of acute kidney injury (AKI), NGAL might soon emerge as an early marker for AKI. It appeared to fulfill many characteristics of an appropriate 'real-time' biomarker for AKI detection<sup>(4)</sup> and its level was a useful prognostic tool with regard to the prediction of renal replacement therapy initiation<sup>(5)</sup>.

Recent evidence also suggests its role as a biomarker in a variety of other renal and non-renal conditions<sup>(6)</sup>. Recently, clinical nephrologists have discovered that NGAL can be a predictor of the progression of chronic renal

diseases<sup>(7)</sup>. NGAL can be also used as a biomarker beyond the confines of nephrology. For example, Serum NGAL could be valuable in the detection of early renal impairment after coronary angiography<sup>(8)</sup> and may play a crucial role in vascular remodeling and plaque instability during the development of atherosclerosis<sup>(9)</sup>.

The aim of the present work is to evaluate circulating NGAL as a biomarker of renal anemia in chronic hemodialysis patients.

## Subjects and Methods

This study is conducted on 40 HD patients receiving hemodialysis in Ain Shams University Hospital compared with 20 healthy individuals. They are divided into 2 groups:

I) Group 1 (healthy control group): includes 20 healthy control individuals with hemoglobin (Hb) level  $> 13\text{g/dL}$  <sup>(10)</sup>.

II) Group 2: includes 40 ESRD patients on regular HD thrice weekly 4-hour sessions for more than 6 months. They had been on recombinant erythropoietin therapy for at least 6 weeks in the dose of 4000 IU thrice weekly<sup>(11)</sup>. These patients are classified into:

**Group 2 (A):** 20 cases with Hb level  $< 11\text{ g/dl}$

**Group 2 (B):** 20 cases with Hb level  $< 13\text{ g/dl}$  and  $\geq 11\text{ g/dl}$

All the individuals included in the study are subjected to:

I) Full medical history and examination with special stress on presence of the cause of end stage renal disease (ESRD) leading to dialysis

II) Laboratory Investigations: Peripheral venous blood samples are taken and 3 biochemical parameters measured, according to standard methods used in the routine clinical laboratory as follows:

A) Hb level: It is estimated using fully automated auto-analyzer "Beckman Coulter" LH 750. It is used for classification of the study groups

B) Iron status is assessed by measuring:

a) Total serum iron using OLYMPUS analyzer<sup>(12)</sup>

b) Serum ferritin is determined quantitatively by a Microplate Immunoenzymometric Assay <sup>(13)</sup>

c) Total Iron Binding Capacity (TIBC) is assayed with the same method used for iron determination then transferrin saturation (TSAT) is calculated as follows:

$$\text{TSAT \%} = \text{Serum Fe} \times 100 / \text{TIBC}$$

C) C-reactive protein (CRP): is determined quantitatively by a Microplate Immunoenzymometric Assay<sup>(14)</sup>

D) NGAL is measured in the serum using the ELISA commercially available kit. Its level is expressed as ng/ml.<sup>(15)</sup>

## Results

The present results are statistically analyzed and graphically presented in tables (1-3) and figures (1-10). Compared to the control group, serum iron shows significantly lower levels in the ESRD patients on HD ( $p < 0.001$ ). The % difference was - 39.6% and - 26.5% in group 2A and 2B, respectively (Table 1 and Figure 1). Serum ferritin shows significantly higher levels in group 2A (52.4%,  $p < 0.05$ ) and 2B (88.5%,  $p < 0.001$ ) than the control group (Table 1 and Figure 2). The TSAT% is lower in group 2A (-35.61%,  $p < 0.001$ ) and 2B (-14.2%,  $p > 0.05$ ) compared to the control group (Table 1 and figure 3). The CRP levels are significantly higher in group 2A (366%,  $p < 0.001$ ) and 2B (266%,  $p < 0.001$ ) than the control group (Table 1 and figure 4). As for NGAL, the serum levels are higher in group 2A (44.2%,  $p < 0.001$ ) and 2B (34.9%,  $p < 0.01$ ) compared to the control group (Table 1 and figure 5).

Correlation and linear regression tests, in all the studied subjects, show a highly significant direct correlation ( $p < 0.001$ ) of serum CRP and ferritin ( $r = 0.637$ ,  $p < 0.001$ ). Comparing the Goodness of Fit, the correlation is very strong (" $r^2$ " = 0.401) (Table 2 and figure 6). Table (3) also shows a highly significant inverse correlation ( $p < 0.001$ ) of serum NGAL with Hb level (figure 7), with serum iron (figure 8) and with TSAT % (figure 9) ( $r = - 0.631$ , - 0.578 and - 0.446, respectively). Comparing the Goodness of Fit, the correlation is strong with TSAT % (" $r^2$ " = 0.198) but it is stronger with both Hb and serum iron (" $r^2$ " = 0.398 and 0.334, respectively). The results reveal no significant correlation between serum NGAL and serum ferritin ( $r = 0.244$ ,  $p > 0.05$ ) (Table 3 and figure 10).

**Table (1): shows % difference in the mean serum values of iron, ferritin, TSAT%, CRP and NGAL between HD patients (Group 2A and 2B) versus the control group.**

Serum Parameter	Statistical	Control Group	Group 2A	Group 2B
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	Parameter	(n = 20)	(n = 20)	(n = 20)
Level of Serum Iron (ug/dl)	Mean ± SD	135.3 ± 17.397	81.7 ± 23.49	99.5 ± 36.515
	level of significance & % of difference from control group		*** - 39.6 %	*** - 26.5 %
Level of Serum Ferritin (ng/ml)	Mean ± SD	75.45 ± 23.234	115 ± 61.529	142.2 ± 36.557
	level of significance & % of difference from control group		* 52.4 %	*** 88.5 %
Percentage of Transferrin Saturation (TSAT %)	Mean ± SD	34.25 ± 6.015	22.05 ± 5.907	29.4 ± 11.408
	level of significance & % of difference from control group		*** - 35.6 %	† - 14.2 %
Serum C-reactive Protein (CRP, ug/ml)	Mean ± SD	2.03 ± 1.006	9.458 ± 3.313	7.44 ± 2.581
	level of significance & % of difference from control group		*** 366 %	*** 266 %
Level of Serum NGAL (ng/ml)	Mean ± SD	145.35 ± 38.348	222.6 ± 24.058	208.15 ± 24.225
	level of significance & % of difference from control group		*** 44.2 %	** 34.9 %

† = Non-Significant difference from the control group ( $p > 0.05$ )

\* = Significant difference from the control group ( $p < 0.05$ )

\*\* = Highly significant difference from the control group ( $p < 0.01$ )

\*\*\* = Very highly significant difference from control group ( $p < 0.001$ )

**Table (2): Correlation between serum C-reactive protein (CRP, ug/ml) and serum ferritin level (ng/ml) in all studied subjects.**

	CRP (ug/ml)		
	R	r <sup>2</sup>	P
Serum ferritin (ng/ml)	<b>0.637***</b>	<b>0.401</b>	<b>&lt; 0.001</b>

**Table (3): Correlation between Neutrophil Gelatinase-Associated Lipocalin (NGAL) level and the parameters describing the iron status in all studied subjects.**

	NGAL (ng/ml)

	R	r2	P
Hb (g/dl)	- 0.631***	0.398	< 0.001
Serum total iron (ug/dl)	- 0.578***	0.334	< 0.001
TSAT %	- 0.446***	0.198	< 0.001
Serum ferritin (ng/ml)	0.244 †	0.059	> 0.05

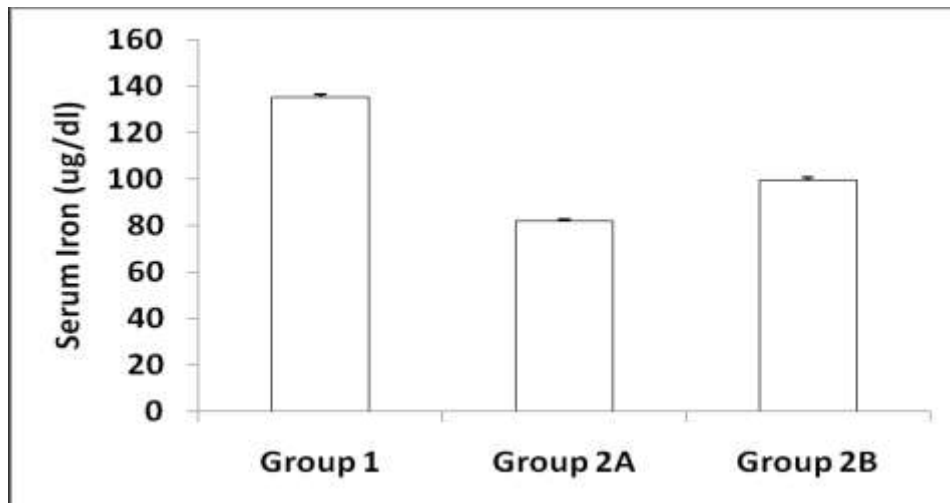


Figure (1): shows the mean values of serum iron in HD patients (Group 2A and 2B) versus the control group.  
 \*\*\* = Very highly significant difference from control group (p < 0.001)

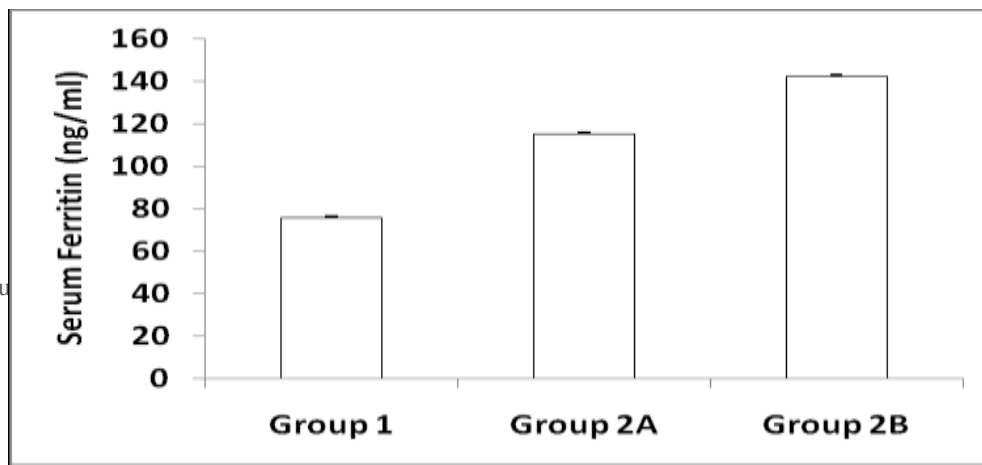


Figure (2): shows the mean values of serum ferritin in HD patients (Group 2A and 2B) versus the control group. (group 2A)\* = Significant difference from the control group ( $p < 0.05$ )  
\*\*\* = Very highly significant difference from control group ( $p < 0.001$ )(Group 2B)

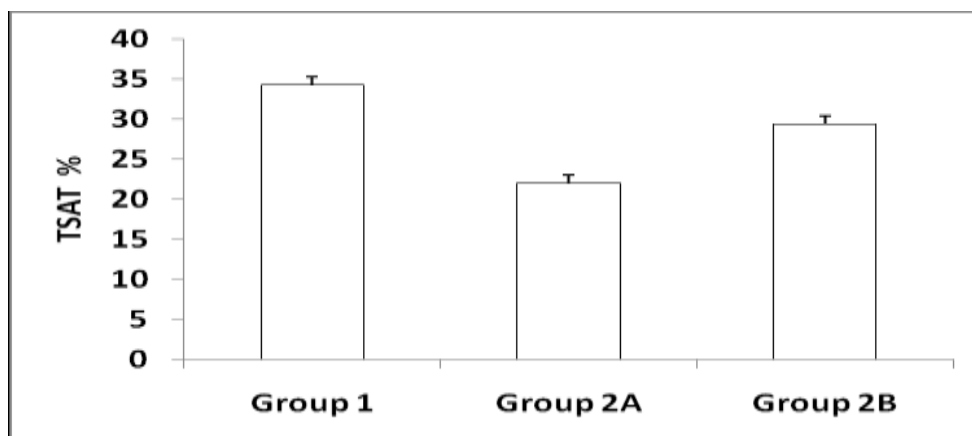


Figure (3): shows the mean values of TSAT% in HD patients (Group 2A and 2B) versus the control group. (Group 2B)† = Non-Significant difference from the control group ( $p > 0.05$ )  
\*\*\* = Very highly significant difference from control group ( $p < 0.001$ )(Group 2A)

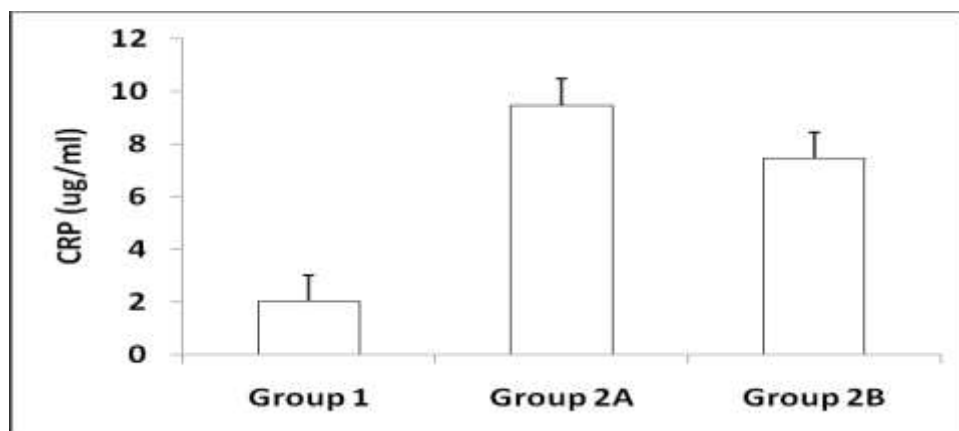


Figure (4): shows the mean values of serum CRP in HD patients (group 2A and 2B) versus the control group. \*\*\* = Very highly significant difference from control group ( $p < 0.001$ )

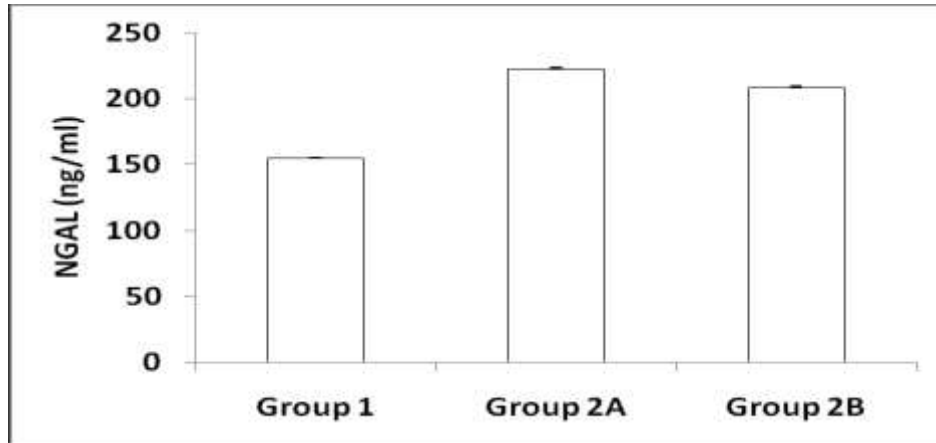


Figure (5): shows the mean values of serum NGAL in HD patients (group 2A and 2B) versus the control group. (Group 2B)\*\* = Highly significant difference from the control group ( $p < 0.01$ )  
\*\*\* = Very highly significant difference from control group ( $p < 0.001$ )(Group 2A)

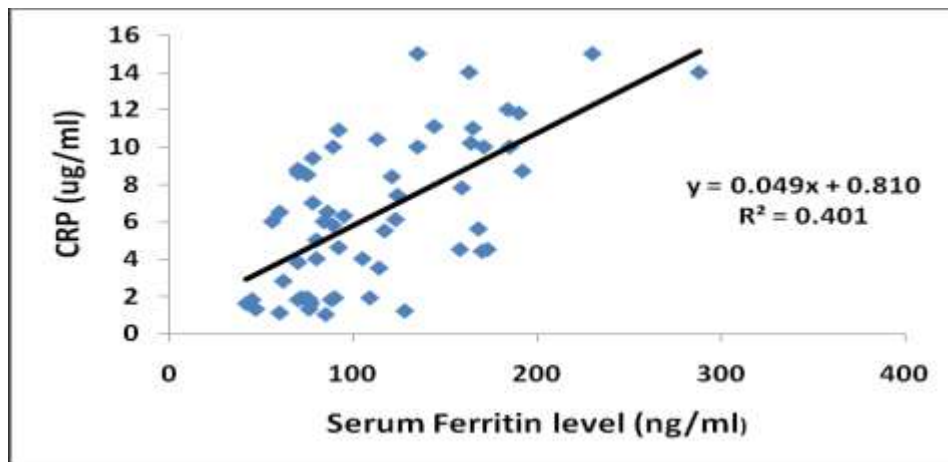


Figure (6): Correlation between serum CRP (ug/ml) and serum ferritin level (ng/ml) in all studied subjects.

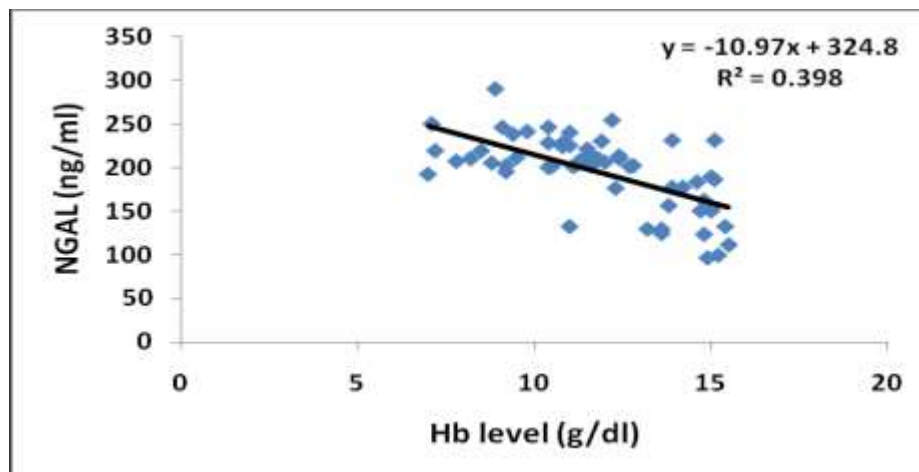


Figure (7): Correlation between serum NGAL (ng/ml) and hemoglobin level (Hb, g/dl) in all studied subjects.

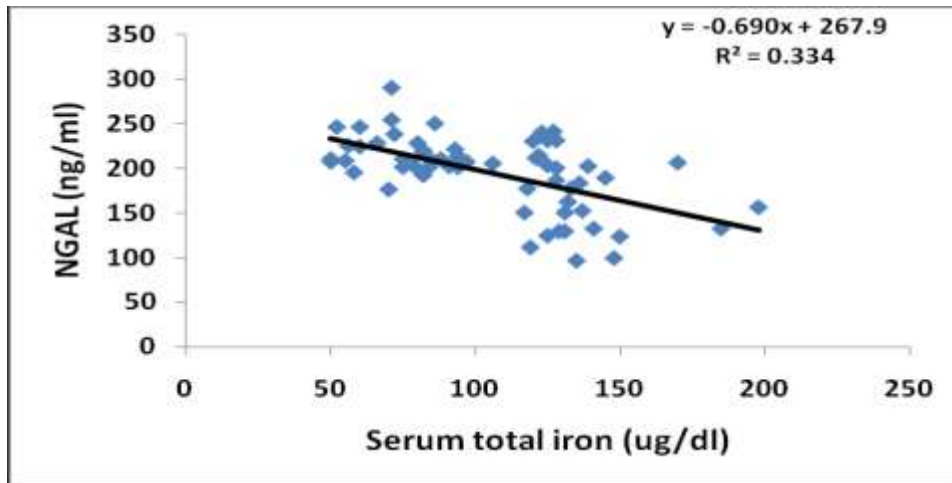


Figure (8): Correlation between serum NGAL (ng/ml) and serum iron (ug/dl) in all studied subjects.

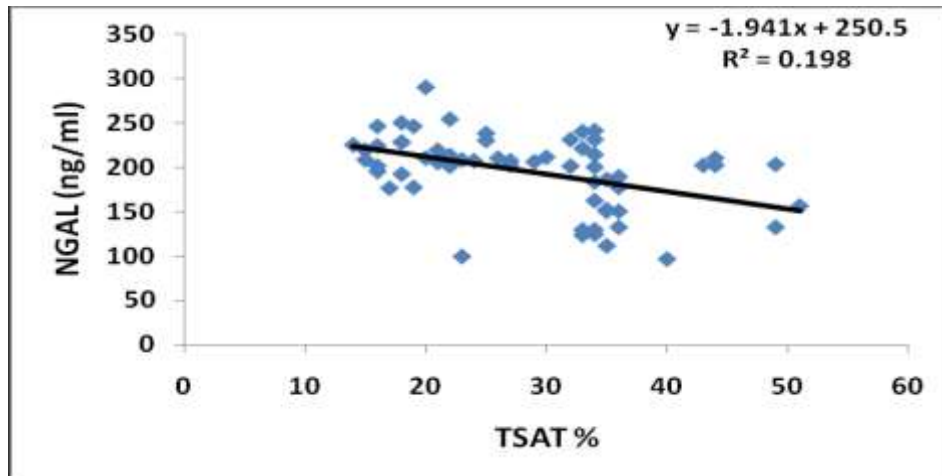
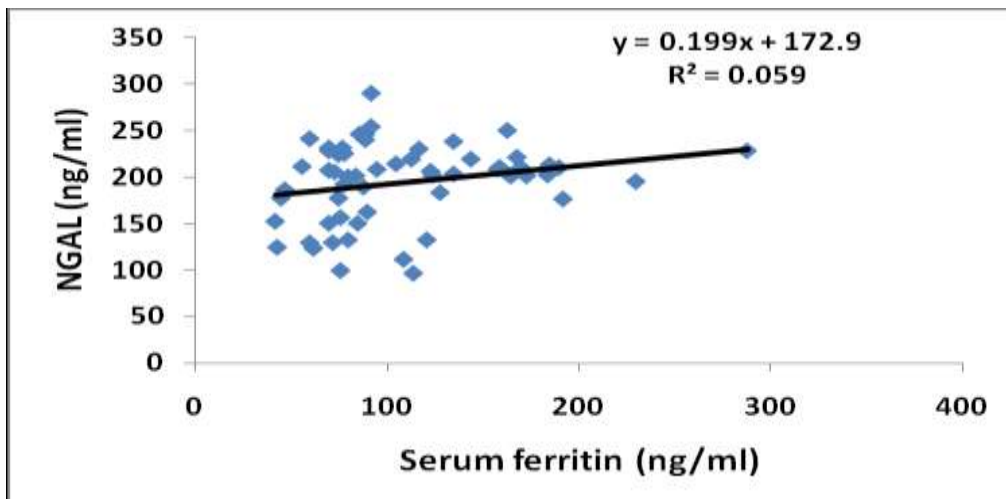


Figure (9): Correlation between serum NGAL (ng/ml) and transferrin saturation (TSAT%) in all studied subjects.



*Figure (10): Correlation between serum NGAL (ng/ml) and serum ferritin (ng/ml) in all studied subjects.*

## Discussion

The present results show that the mean serum iron level is significantly lower ( $p < 0.001$ ) in the ESRD patients on HD (group 2A and 2B) than the control group. These results are in agreement with the findings of *Silverberg (2010)*<sup>(4)</sup> who reported that anemia is frequently observed among chronic HD patients and considered iron deficiency a consequence of frequent blood sampling, GI bleeding, dietary restrictions and/or decreased enteric absorption.

In this study, the mean serum ferritin levels are significantly higher in the ESRD patients on HD than the control group ( $p < 0.05$  and  $< 0.001$  in group 2A and 2B, respectively). On the contrary, the mean values of TSAT% are lower in the ESRD patients on HD than the control group ( $p < 0.001$  and  $> 0.05$  in group 2A and 2B, respectively).

It has been reported that similar changes (elevated serum ferritin and low TSAT%) are often also seen in patients with inflammatory processes associated with epoetin hyporesponsiveness. While a CRP level diagnostic of inflammation-related epoetin hyporesponsiveness in dialysis patients has not been established, a level above 20 mg/l would be very suggestive of an active, underlying inflammatory process<sup>(16)</sup>.

Serum ferritin and the TSAT have been considered primary tools in the assessment of iron status in nephropathic subjects<sup>(2)</sup>. More recently, however, *Rambod et al. (2008)*<sup>(3)</sup> said that ferritin is an acute-phase reactant, is markedly influenced by malnutrition and has important gender differences, thus making it a less than ideal tool for identifying iron deficiency. For example, the presence of inflammation can explain the apparently paradoxical coexistence of high ferritin ( $> 500$  ng/ml) and low TSAT ( $< 25$  %) levels frequently found in HD patients.

The present results regarding the mean values of CRP go in parallel with those data. Our results reveal that CRP levels are significantly higher in the ESRD patients on HD (group 2A and 2B) than the control group ( $p < 0.001$ ). The high CRP level explains the high serum ferritin levels in the two patient groups, considering serum ferritin as an acute-phase reactant which is increased during inflammation that frequently associates HD.

The present results show significantly higher values of the mean serum NGAL levels in the ESRD patients on HD than the control group ( $p < 0.001$  and  $< 0.01$  in group 2A and 2B, respectively). Although the main physiologic font of NGAL is represented by neutrophils (in accordance with its function as an innate anti-bacterial factor), it is now widely accepted that this protein is a true acute-phase factor that can be released by virtually almost every injured tissue, often becoming a marker of disease severity<sup>(17)</sup>. For instance, in subjects with CKD, increased serum and urinary NGAL levels correlate with residual renal function<sup>(18)</sup> and a single measurement of NGAL after treatments potentially detrimental to the kidney (e.g. contrast administration, cardiac surgery) becomes useful in the early prediction of incipient acute kidney injury (AKI)<sup>(19)</sup>. *Haase et al. (2009)*<sup>(5)</sup> analyzed data from 19 studies in 8 countries involving 2,538 patients, of whom 487 (19.2%) developed AKI and reported that NGAL level was a useful prognostic tool with regard to the prediction of renal replacement therapy initiation and in-hospital mortality.

In our HD patients, we can speculate that, overall, the rise of NGAL level belongs to a wide panel of responses to the systemic inflammation associated with chronic HD treatment; this would be supported by the significantly higher levels of CRP and ferritin in our two HD patient groups when compared to the control group. In accordance with our results, clinical nephrologists have recently discovered that NGAL can be a predictor of the progression of chronic renal diseases<sup>(7)</sup>. In an earlier study, HD patients showed increased NGAL values compared to healthy subjects. Direct significant correlations were reported between NGAL and hsCRP, serum ferritin and ESR, three well-known indexes of systemic inflammation<sup>(20)</sup>.

The present study shows strong direct correlation between serum CRP and ferritin ( $p < 0.001$ ), both considered well-known indexes of systemic inflammation. On the contrary, there is no significant correlation between NGAL and ferritin ( $p > 0.05$ ). These results could be indicative of a strong confounding effect played by inflammation on



ferritin level more than NGAL. This is supported by the recent criticism concerning the effective utility of serum ferritin measurement in correctly evaluating iron status in HD patients with inflammation<sup>(21)</sup>.

The present results show strong inverse correlation ( $p < 0.001$ ) between NGAL and Hb, and between NGAL and iron. Recently, clinical nephrology has discovered NGAL, a small inflammatory cytokine, as one of the most promising biomarkers in the diagnostic field of kidney diseases. NGAL can predict the early onset of AKI after treatments potentially detrimental to kidney, as well as the progression of chronic renal diseases.<sup>(7)</sup> However, other parallel studies have uncovered the unexpected property of this factor to physiologically inhibit human erythropoiesis: NGAL, produced and released by the same erythroid precursors in the bone marrow, blocks their growth and differentiation and induces apoptosis through an inhibitory auto-feedback<sup>(22)</sup>. These results go parallel with our results of the inverse correlation between NGAL and both Hb and serum iron.

Also, in line with these findings, several systemic diseases associated with secondary anemia, such as chronic renal failure, chronic inflammation and cancer, were known to induce a dramatic increase in circulating NGAL levels. This may represent a further, important cause of the development and worsening of anemia itself. NGAL may thus become an alternative therapeutic target for improving the treatment of secondary anemia related to these conditions<sup>(23)</sup>.

In a recent murine model<sup>(24)</sup>, NGAL tissue levels were markedly increased in different experimental models of induced anemia e.g. after phlebotomy or iron deprivation; this protein may therefore play a physiological role during increased iron utilization and mobilization from stores. These observations raised the possibility that NGAL might also be involved in the maintenance of the iron balance in HD patients. Findings reported in the present work effectively confirm this hypothesis, as NGAL levels are found to be strongly negatively correlated with TSAT ( $p < 0.001$ ), one of the two main laboratory references of iron stores proposed in the K/DOQI guidelines<sup>(2)</sup>.

## Conclusion

The use of NGAL as a biomarker in assessment of renal anemia among HD patients is probably of great potential and is superior and more specific than ferritin. Furthermore, its significant correlation with s.iron and TSAT suggests that NGAL is also a potentially useful biomarker for the management and guidance of iron therapy in HD patients. However, these findings are preliminary and there is a need to evaluate the real effect of chronic inflammation on circulating NGAL levels, and to make an effective cost-to-benefits analysis.

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