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NGAL as a biomarker in chronic kidney disease

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***Abstract*---**The current study was performed with 65 patients with Chronic kidney disease, their mean age (44.38±14.59) years, who have visited the physician's consultancies and the kidney disease unit at Al-Sadder Teaching hospital in Al-Najaf, Iraq, and 25 persons as a healthy control group their mean age (48.68±16.37) years. The study was conducted from September 2021 to May 2022. In this study, serum NGAL levels were measured in patients and healthy groups. It also assays the relationship between levels of these biomarkers with some hematological and biochemical parameters RBCs, Hb, PCV, Creatinine, Urea, GFR. The results revealed that there was a Significant decrease with mean± SD of RBCs, HGB, as well as PCV, (3.36±0.69 X106/ mm3), (8.99±1.76mg/dl), (28.47±4.2%) respectively of patients with kidney disease as compared with healthy groups (4.55±0.47 x 106mm3), (14.28±1.43 mg/dl), and (40.11±3.84 %) respectively. The results also shows significant difference (p-value<0.05) decreased with mean± SD of eGFR in Kidney failure patients (13.42±13.83 ml/min/1.73 m2) as compared with healthy control group (114.12±12.49 ml/min/1.73 m2).The results also indicated that there is significant difference (p-value<0.05) increased in the mean level of urea (156.6±53.97 mg/dl), creatinine (8.57±4.25mg/dl), and blood sugar(177.92±74.11), in patients with Kidney failure as compared with healthy control group (26.76±6.26 mg/dl) (0.7±0.12 mg/dl),((93.99±10.75), respectively. The results also indicated there was significance difference (p<0.01) increase in serum NGAL (5.93±1.17) in patients with CKD as compared with healthy control group (1.83±0.59).

***Keywords*---**NGAL, neutrophile gelatinase, lipocaline, chronic kidney disease.

Introduction

Chronic Kidney disease (CKD) is a condition in which kidney function gradually deteriorates. The filtration function of the kidneys gradually deteriorates over months or years and is typically permanent. Current international recommendations describe this syndrome as impaired kidney function manifested by a glomerular filtration rate (GFR) of less than 60 ml/min per 1.73 m2 or indicators of kidney damage for at least three months, regardless of the underlying etiology(Almaawi, 2021). Many people are asymptomatic or have no symptoms at all, such as itching, fatigue, or a loss of appetite. GFR, which is assessed with exogenous markers (e.g., DTPA, iohexol) or calculated using equations, is the best indication of general renal function (Rusul 2020). After the age of 40, the typical rate of reduction in GFR in this group is 0.75 to 1 ml/min/year. (Sharif et al.,2017). Proteinuria has been linked to an elevated risk of chronic renal disease progression and mortality. Anemia produced by decreased erythropoietin production by the kidney, iron insufficiency and reduced RBC survival, and mineral bone disease caused by inadequate vitamin D, phosphate, and calcium metabolism are all complications. (Rusul 2020; Johnson et al.,2013). Symptoms of CKD are usually not obvious in the early stages. The initial symptom of the illness is a significant decrease in renal function. The course of chronic kidney disease can be slowed and consequences reduced if it is detected early (stages 1,2,3).(Rysz et al., 2017) Kidney damage is extensive in stages 4 and 5, leading to end-stage renal failure. CKD is currently diagnosed based on serum creatinine (SCR) and blood urea levels; however, sCr has been demonstrated to have little predictive value. (So, 2018) . The evolution of epigenetics, genomes, transcriptomics, metabolomics, and proteomics will allow for the identification of new biomarkers in kidney disease. These biomarkers include asymmetric dimethylarginine (ADMA), uromodulin, symmetric dimethylarginine (SDMA), kidney injury molecule-1 (KIM-1), miRNA, neutrophil gelatinase-associated lipocalin (NGAL), ncRNA, and lincRNA biomarkers, as well as proteomic and metabolic biomarkers. (Rysz et al., 2017). The study aimed to asses serum NGAL in CKD patients and studying the correlations between this biomarker and biochemical and hematological parameters

Materials and Methods

Subject of patients

65 samples collected from males patients with CKD their mean age (44.38±14.59) years, who have visited the physician's consultancies and the kidney disease unit at Al-Sadder Teaching hospital in Al-najaf, Iraq, some of them were accepted for having other diseases such as heart diseases, uncontrolled hypertension, diabetes Mellitus, cancer, Inherited kidney conditions, and Psoriasis. Patients with the following illnesses were excluded from the current study: liver diseases (including positive HBsAg and anti-HCV Ab), chronic inflammation (including inflammatory bowel disease), and thyroid disorders.

Control Group

Twenty-five volunteers of males as a control group were recruited from people working in these hospitals and science faculty institution, which had no diseases such as diabetes, cardiovascular disorders, anemia, and kidney diseases.

Laboratory Measurements

Five ml of venous blood was collected from all participants in the early morning and before one hour of hemodialysis session, then divided into 1.5 ml put in EDTA-containing tubes for CBC, and 3.5 ml in gel tube and centrifuged for serum separation the serum then divided into two and saved in Eppendorf tubes and stored at deep freezer until the analyses of biochemical parameters and biomarkers.

Measurement of hematological parameters

The blood samples were collected in EDTA tubes and complete blood count (CBC) indices were directly measured by automated hematological equipment using Aboot TDX analyzer (U.S.A) in the hematology laboratory of AL-Sadder Medical city, Aboot TDX is an automated hematology analyzer using impedance technology for a complete blood cell count (CBC).

Measurement biochemical parameters

- **Estimation of creatinine**
  Creatinine was estimated by the Jaffe reaction, a calorimetric procedure in which creatinine forms a yellow orange complex in alkaline solution with picric acid. This colored complex is determined photometrically. The intensity of produced colored is directly proportional to the amount of creatinine in the sample.
- **Estimation of urea**
  Urea was measured by diacetyl monoxime colorimetric method and Berthelot reaction. In this method the urea is converted to ammonia by an enzyme called urease. ammonia produced is combined with 2-oxoglutarate and NADH in the presence of glutamate dehydrogenase (GDH), which yields L-Glutamate and NAD. The decrease in NADH absorbance is proportional to the urea concentration. (Appendix A) GFR were calculated with specific equation based on creatinine levels and during the follow-up (Levey et al., 2010).
- **Estimation of serum glucose**
  Trinder Method. Glucose is oxidized by GOD to gluconic acid and hydrogen peroxide which in conjunction with POD, reacts with chloro4-phenol and PAP to form a red quinoneimine. The absorbance of the colored complex, proportional to the concentration of glucose in the specimen is measured at 500 nm.

Measurement of biomarkers

- **Estimation of NGAL**
  The quantitative sandwich enzyme immunoassay method is used in this assay. A microplate has been precoated with a monoclonal antibody sensitive for NGAL. After incubation, standards and samples are pipetted into the wells, and any NGAL present is collected by the coated antibody. Following thorough washing, a biotin-conjugate antibody specific for NGAL is applied to detect the captured NGAL protein in the sample. Horseradish peroxidase (HRP)-conjugated Streptavidins are then added for signal development, followed by tetramethyl-benzidine (TMB) reagent. After removing any unattached combinations, an enzyme conjugate is introduced to the wells. To cease color development, a sulfuric acid solution is applied, and the color intensity, which is related to the amount of bound protein, is detectable at 450nm.
- **Estimation of Cystatin C**
  The quantitative sandwich enzyme immunoassay method is used in this assay. A microplate has been precoated with a monoclonal antibody specific for Cystatin C. After incubation, any Cystatin C present is captured by the coated antibody. Standards and samples are pipetted into the wells, and any Cystatin C present is captured by the coated antibody. Following thorough washing, a biotin-conjugate antibody specific for Cystatin C is applied to detect the captured Cystatin C protein in the sample. Horseradish peroxidase (HRP)-conjugated Streptavidinis is added for signal development, followed by tetramethyl-benzidine (TMB) reagent. After removing any unattached combinations, an enzyme conjugate is introduced to the wells. To cease color development, a sulfuric acid solution is applied, and the color intensity, which is related to the amount of bound protein, is detectable at 450nm.

Statistical Analysis

The analysis was performed using SPSS v.28 and Microsoft software excel 2019 for graphic. The distribution of continuous variables was analyzed using the Kolmogorov–Smirnov test, in order to assess significant departures from normality. For non-normally distributed parameters, results were presented as median (interquartile range). normal distribution statistical analysis for the differences between group, and Data are expressed as mean ± SD (standard deviations). Using of Independent t tests to compare continuous variables between groups. Whenever the multiple comparisons between groups were performed by one-way ANOVA with Tukey post hoc. Categorical variables were analyzed by the chi-square test. Correlation coefficient analysis was completed with Pearson's or Spearman rank. Binary and nominal multiple regression analyses Discrimination, that is, the model's ability to differentiate between patients and healthy control, was examined using a receiver operating characteristic curve (ROC) to determine the area under curve (AUC). AUROC analysis was also performed to calculate cutoff values, sensitivity, specificity, overall correctness, as well as positive and negative predictive values. Finally, cutoff points were calculated by obtaining the best Youden index (sensitivity +

specificity − 1). Significance of differences was detected at $p<0.05$. (Sullivan, 2017).

Results and Discussion

Hematological parameters in CKD patients

The results revealed significant difference (P-value<0.05) decreased with the mean± SD of RBCs, HGB, as well as PCV, (3.36±0.69 $X10^6$/ mm3), (8.99±1.76mg/dl), (28.47±4.2%) respectively of patients with kidney disease as compared with healthy groups (4.55±0.47 x $10^6 mm^3$), (14.28±1.43 mg/dl),and (40.11±3.84 %) respectively. These were in agreement with prior research conducted by (Shastry, and Belurkar, 2019). They found that changes in hematological parameters tests and a significant decrease in HB, RBCs, PCV, in all kidney disease patients compared with control group, and they noted this decrease in RBC count in the stage of kidney disease is progressed significantly. The results indicated high prevalence of anemia in CKD patients at AL-Sadder teaching hospital with over all prevalence of 65 (95%). A similar finding to this study was displayed in the study conducted in northern Tanzania were the prevalence was 92.4 % among fifty two CKD patients seen Kilimanjaro Christian Medical Centre (Kilonzo, 2010). (Suega et al.,2005) in Indonesia and (Afshar et al.,2010) in Iran showed 73.1 % and 75.0 % prevalence of anemia in pre-dialysis patients, respectively, and in Africa, a Nigerian study recorded 77.5 % and 87 % prevalence of anemia in kidney failure patients (Akinsola et al., 2000; Ijoma et al., 2010). Anemia in kidney failure patients is prevalent due to reduced production of erythropoietin in the kidney due to the reduction of renal mass (Lapin et al.,2002; Maxwell, 2002). EPO is produced by peritubular cells in the kidneys of the adults these cells are located at the tips of the renal pyramids which are very sensitive to ischemia that once sensed leads to an increase production of erythropoietin. The kidneys has the ability to regulate the hematocrit by matching the plasma volume and the red blood cell mass. (Tsagalis, 2011) There are certain other factors that may interfere with erythropoiesis in renal patients, Folate and B12 are required to ensure adequate DNA synthesis especially during the rapid division of erythroblast. (Selhub et al., 2009) In the absence of Iron (blood lose during dialysis, loss from gastrointestinal tract, inadequate food intake), the HB building steps that follow rapid cell division are affected leading to small, poorly hemoglobinized reticulocytes that emerge from the bone marrow and a hypochromic, microcytic anemia (Murali, 2016; Palaka et al. 2020; Tsai et al. 2016). Inflammation that occur commenly in patients with kidney failure inhibits erythropoietin production, impairs the growth of erythroblasts and promotes death of immature erythroblasts. (Koury et al., 2015) Inflammation stimulates hepatic release of hepcidin that promotes iron deficient erythropoiesis by both blocking iron absorption in the gut and iron release from resident macrophages. (Nemeth et al., 2009) Hypothyroidism, uremic toxins, hypersplenism and ongoing infection can reduce the life span of erythrocytes leading to renal anemia. (Tsagalis, 2011).

Estimation of kidney functions

The results in table (1) shows significant difference (p-value<0.05) decreased with mean± SD of eGFR in Kidney failure patients (13.42±13.83 ml/min/1.73 m^2) as compared with healthy control group (114.12±12.49 ml/min/1.73 m2). The results also indicated that there is significant difference (p-value<0.05) increased in the mean level of urea (156.6±53.97 mg/dl), creatinine (8.57±4.25mg/dl), and blood sugar (177.92±74.11), in patients with Kidney failure as compared with healthy control group (26.76±6.26 mg/dl) (0.7±0.12 mg/dl), (93.99±10.75), respectively. These findings were consistent with many previous studies that indicated GFR and proteinuria-albuminuria are the kidney functional parameters currently used to assess KD severity (Huelin et al; 2019). the results of the current study showed that there are a highly significance differences (P≤0.05) Increased levels in serum urea and creatinine values in kidney failure Patients as compared to the healthy control group, these studies are in agreement with previous studies (Ahssan et al ., 2010; Schaalan et al., 2016; Abhisek et al., 2016).the results also show significance difference (p<0.01) decrease in GFR levels in patients with kidney failure as compared with healthy control group, GFR declines with increasing age, which is a classic risk factor for renal failure patients (Levey et al., 2012).As a result, renal failure patients are more common in adults than in children. In this investigation, the primary underlying diseases for renal failure patients were identified as hypertension (HTN) and diabetes mellitus (DM).As a result, an active screening program for patients, particularly those at risk, such as those with HTN and diabetes, is in place, with the target of slowing the development of AKI and CKD (Al-wazni, 2017).Reduced urea filtration may be the cause of high uric acid levels in renal failure, resulting in a reduction in (GFR) in Kidney failure patients. The measurement of urea level as an indication of renal function is based on the observation that serum/plasma urea level reflects GFR. The current investigation confirmed that the GFR level in patients fell significantly (p<0.01) when compared to the healthy group, and this result agrees with previous findings. (Bobulescu et al ., 2012).

Biomarkers

NGAL as a biomarker of kidney disease patients

The study revealed that patients with kidney failure show significance difference (p<0.0001) increase in Serum levels of NGAL in kidney failure patients (5.93±1.17) as compared with healthy control group (1.83±0.59) that explained in table (1). This was compatible with(Moriya et al 2017; Rusul, 2020)) a study indicated that plasma NGAL was an important biomarker of interstitial lesions in CKD patients.Researchers also indicated that NGAL could have been used to estimate the end stage of risk levels for CKD. There was evidence that NGAL may also be used as a mediator of CKD progression; (Viau et al; 2010) Previously, (Mishra et al; 2003) NGAL was identified to be one of the first highly active genes and proteins in tubular epithelial cells in the distal nephron, and that it was released from these cells following tissue damage, such as ischemic renal injury. More study has showed that there are several molecular forms of NGAL in urine, including a monomeric form produced by kidney tubular epithelial cells and a dimeric form produced by neutrophils. As a result of this variation, NGAL has the

potential to increase the specificity of NGAL as a renal biomarker (Cai et al; 2010). NGAL, is also defined as siderocalin, lipocalin-2 or lipocalin, is prevalent at multiple location, In the human Leukocytes were the main source of its expression originally isolated from neutrophils in addition to other tissues such as liver, epithelial cells, kidney particularly from nephrons loop of Henle and the collecting ducts (Wasung, et al. 2015; De Silva, et al; 2021). Diabetic nephropathy (DN) is a common diabetes complication caused by adverse renal tubular injury, which plays a critical part in the development and progression of DN, and renal tubular epithelial cell apoptosis contributes to kidney dysfunction, elevated incidence of serum creatinine, blood urea nitrogen, urine total protein/urine creatinine, and microalbuminuria, and decreased creatinine clear rate. (dabla et al; 2010). Several studies have demonstrated the importance of urine NGAL as a predictive biomarker in diabetic nephropathy, but few have investigated the role of serum NGAL in these individuals (Fu, 2012). In DMT2 patients, NGAL was not significantly raised in the early stages of diabetic nephropathy with Normal-Albuminuria and microalbuminuria. Another cross-sectional research of DMT1 individuals found no link between urine NGAL levels and GFR (Kim et al; 2012).

Serum NGAL level correlated with other studied parameters in CKD patients

Our study revealed high positive correlation between serum NGAL and creatinine ($r=0.456$, p-value<0.001) as showed in figure (1). The results also indicated there was positive correlation between NGAL and Urea as showed in figure (2) ($r=0.498$, P-value<0.001). the results also show high negative correlation between serum NGAL and eGFR ($r=0.321$, $p<0.01$)as showed in figure (3). The results in table (2) show negative correlation between serum NGAL and RBCs ($r=-0.325$, $p=0.041$), HGB ($r=-0.299$,$p=0.042$),PCV($r=-0.261$,$p=0.036$), MCH ($r=-0.302$, $p=0.01$), MCHC ($R=-0.290$, $P=0.019$)and Neutrophils($r=-0.265$, $p=0.033$). the results also showed no significance correlation between NGAL and MCV ($R=-0.141$, $P=0.262$) (Rusul, 2020 ; Guo et al; 2020). Malyszko and his college indicated that serum NGAL is strongly correlated with serum creatinine ($r=0.78$) (Malyszko et al., 2009). The results indicated that serum level of NGAL was moderately positive correlated with neutrophils and this is agreed with (Mårtensson et al., 2013)as showed in table (2) they found that Elevated NGAL was associated with sepsis independent of level of acute renal dysfunction. A cut-off value of 98 ng/mL distinguished sepsis from systemic inflammation with high sensitivity and specificity. Anemia is the most common kidney failure symptoms. In addition, many other systemic diseases, such as chronic inflammation, are associated with secondary anemia. Another common characteristic of the aforementioned diseases is significant high-serum NGAL levels. This may indicate an additional, significant exploration of the anemia- NGAL relation. Several studies have shown that NGAL role in RBC physiology and pathophysiology through it promotes apoptosis and prevents the differentiation of progenitor erythroid cells thus act a key role in anemia (Yazdani et al; 2014; Ismail et al; 2015). (Miharada et al.,2005) reported that after exogenous administration of recombinant NGAL in mice, a relatively large proportion of the protein was specifically bound to receptors on the surface of marrow cells. In cellular models in vitro, about 3 hours after administration of NGAL to cultures of immature erythroid precursors, an increased activation of caspase 3/7 and the induction of apoptosis were reported. The NGAL-induced inhibition of erythropoiesis, through the stimulation of apoptosis and arrest of

differentiation in immature erythroids, was also confirmed by the in vivo induction of hemolytic anemia through the administration of phenylhydrazine, which dramatically reduces NGAL levels, particularly at the medullary level, thus leading to a marked increase in erythropoiesis. However, the administration of recombinant NGAL immediately after the induction of anemia markedly inhibits the recovery of hematopoietic function. Therefore, when red blood cells are in greater demand, the immature erythroid cells probably activate mechanisms in order to “escape” the effects of NGAL. This would occur through reduced NGAL synthesis in response to factors such as IL-3 (Devireddy et al., 2005), and the induction of cell survival systems (e.g. BCL-XL cascade) culminating cell growth and differentiation.

Table 1
Biochemical and hematological parameters in CKD patients compared with the healthy control

Groups / parameters	Mean ± SD		p-value
	CKD Patients n=65	Healthy control n=25	
Age (year)	44.38±14.59	48.68±16.37	0.230
Dialysis duration (m) #	6 (0-30)	0	NA
wieght (kg)	70.22±17.89	75.56±9.15	0.159
GFR (ml/min/1.73 m^2) #	13.42±13.83 7 (5-18) #	114.12±12.49 114 (103-125)#	0.0001 *
Urea (mg/dl)	156.6±53.97	26.76±6.26	0.0001 *
Creatinine (mg/dl)	8.57±4.25	0.7±0.12	0.0001 *
RBC (X10^6/ mm^3)	3.36±0.69	4.55±0.47	0.0001 *
HGB (mg/dl)	8.99±1.76	14.28±1.43	0.0001 *
P.C.V. %	28.47±4.2	40.11±3.84	0.0001 *
B. sugar (mg/dl)	177.92±74.11	93.99±10.75	0.0001 *
NGAL (ng/ml)	5.93±1.17	1.83±0.59	0.0001 *

*significant difference at p-value <0.05. # median (IQR) inter quartl rang. NA not implicated

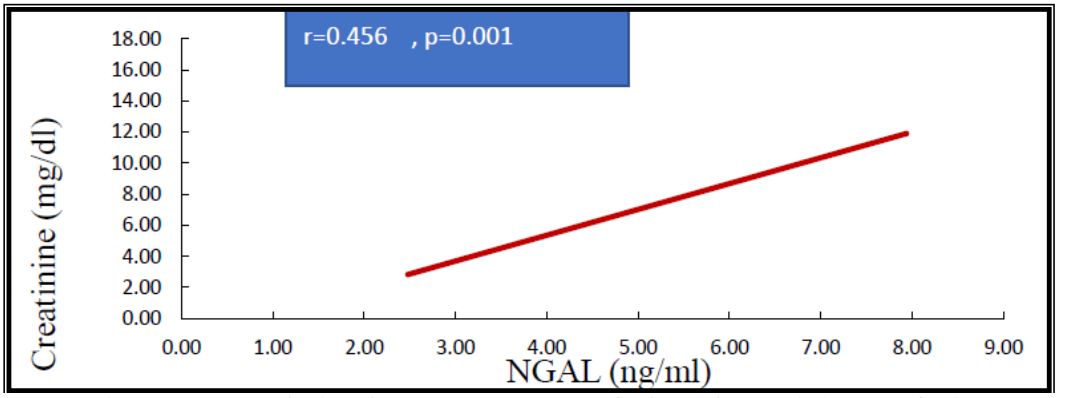

Figure 1. Correlation between NGAL (ng/ml) and creatinine (ng/ml)

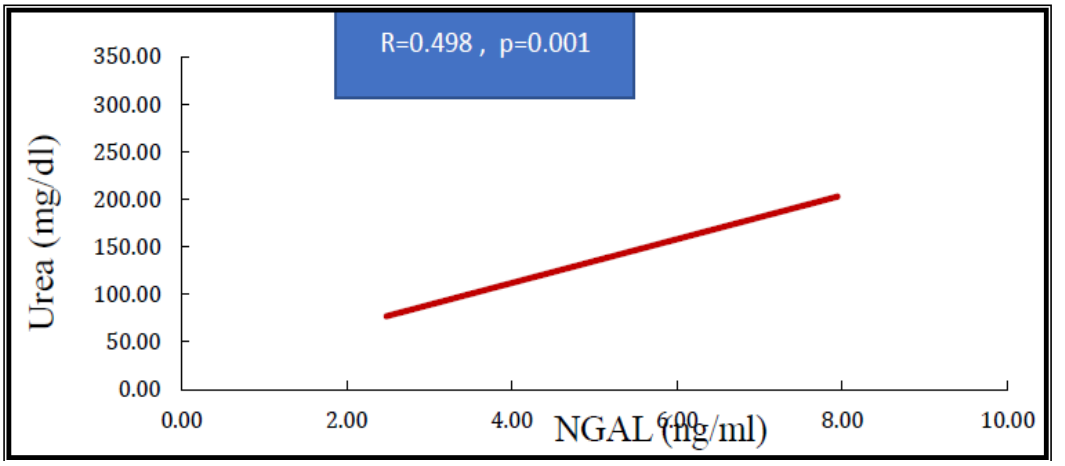

Figure 2. Correlation between NGAL (ng/ml) and urea (ng/ml)

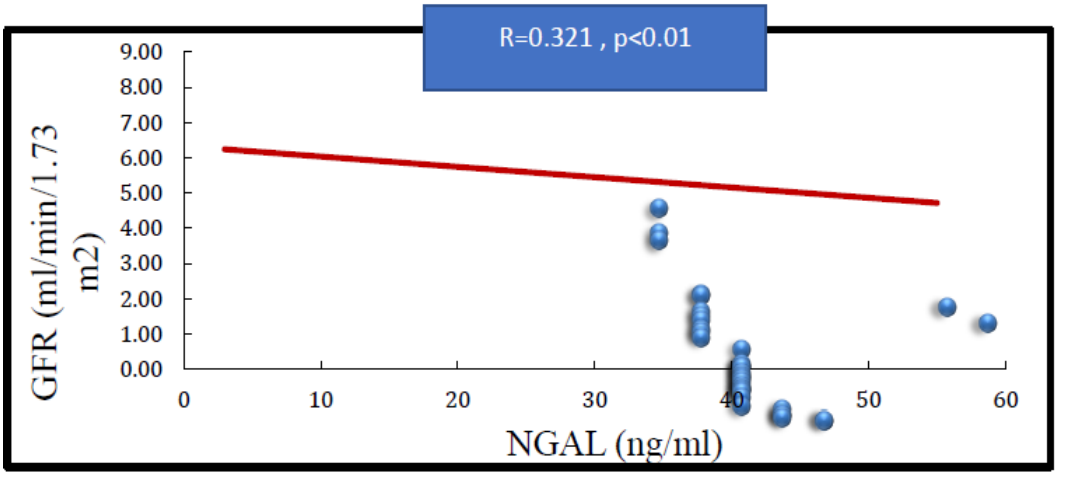

Figure 3. Significant negative correlation of GFR with serum NGAL level in CKD patients

Table 2
Correlations between NGAL and different hematological parameters

		NGAL (ng/ml)
RBC (X10^6/ mm3)	R	0.325-
	P-VALUE	0.041
	N	65
HGB (mg/dl)	R	-0.299-
	P-VALUE	0.042
	N	65
P.C.V. %	R	0.261*-
	P-VALUE	0.036
	N	65
MCV (fL)	R	-0.141-
	P-VALUE	0.262
	N	65
MCH (pg)	R	-0.302-*
	P-VALUE	0.015
	N	65
MCHC (g/dl)	R	-0.290-*
	P-VALUE	0.019
	N	65
NEUT (X103/mm3)	R	-0.265-*
	P-VALUE	0.033
	N	65

* Significance difference at p-value <0.05

Conclusions

There was decreased of eGFR in Kidney failure patients as compared with healthy control group and there is significant increased in the mean level of urea, creatinine and blood sugar in patients with Kidney failure as compared with healthy control group. The results also indicated there was significance increase in serum NGAL in patients with chronic kidney disease as compared with healthy control group.

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