# Differential Expression of Proinflammatory Cytokines IFN-γ and TNF-α in CKD Patients from South India.

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**Research Article**

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# Differential expression of proinflammatory cytokines IFN-γ and TNF-α in CKD patients from south India

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## Abstract

**Objective:** To explore the proinflammatory cytokine expression from chronic kidney disease patients (CKD) with its secondary complications. **Methods:** A total of 133 CKD patients and 149 healthy controls were evaluated for TNF-α and IFN-γ cytokine mRNA expression by qRT- PCR methods. **Results:** We found upregulated expression for TNF-α (FC: 2.5) and IFN-γ (FC:1.76) in pooled CKD patients when compared to controls. The expression profile for TNF-α and IFN-γ was 2.6 and 1.71 fold respectively for dialysis patients. However, in Non-Dialysis patients, a down regulated expression for TNF-α (FC: 0.19) and upregulated expression for IFN-γ (FC:1.6) were noticed. The IFN-γ and TNF-α expression level was 2.02 and 1.79 fold respectively for CKD patients with diabetes. Whereas in non-diabetic CKD patients, the IFN-γ and TNF-α expressions were 1.79 and 1.87 fold respectively. When we grouped the data based on with and without complications, we found a significant upregulated expression for IFN-γ (FC:1.64) and down regulated expression for TNF- α (FC:0.65) were observed in without complications. A significant upregulated expression were observed for IFN-γ (FC:1.82) and TNF- α (FC:2.27) in with complications. We have observed a significant positive correlation between TNF-α and IFN-γ

(R=0.620; p< 0.0001). Analysis of data showed negative correlation between eGFR and Creatinine in Dialysis and Non-Dialysis group of patients. The disease severity progression showed that, 84.2% (n=112) of individuals fall under <15 eGFR. **Conclusion**: Increased TNF-α and IFN-γ expression suggests that Th1 cells are involved in CKD inflammation and its disease pathogenesis.

Key words: Cytokine, expression, Interferon-γ, Tumor necrosis factor-α, Fold Change

## Introduction

Chronic kidney disease (CKD) is one of the important public health problems worldwide. The CKD is defined based on the presence of kidney damage with reduced functions which longer than 3 months [1-2]. The rapid increased of CKD in worldwide is associated with increased incidence of diabetes [3], Obesity, hypertension [4], age-related decline renal functions and primary renal disorders [5]. The burden of CKD is increasing globally, and is estimated to become the 5th most common cause of years of life lost globally by 2040. Approximately, >850 million people affected CKD in worldwide and mortality rate of 1.2 million (approx.) per year [6-7]. The incidence of CKD was varying from different regions ranges from 1% to 17% which is reported recently from the International Society of Nephrology's Kidney Disease Data Center [8]. The high levels of CKD with unknown etiology (CKDu) were seen in Andhra Pradesh, Odisha, and Goa, which may be due to chronic interstitial nephropathy with insidious onset and slow progression [9]. The increased prevalence of CKD will lead to end-stage renal disease (ESRD) followed by renal replacement therapy (RRT) [10, 11]. The risk factors like poor sanitation, poverty, pollutants, overcrowding, water contaminations, known and unknown nephrotoxins which may lead to glomerular and interstitial kidney diseases. The secondary complications such as diabetes and hypertension are the principal risk factors for CKD4, and CKD is associated with cardiovascular morbidity and mortality [12-13], in early stages and also in young patients [14].

Cytokines are released from activated or injured kidney cells, which in turn activate to specific sites of injury in kidney failure patients. Kidney proximal tubule cells produce proinflammatory cytokines in response to lipopolysaccharide (LPS) [15], or albumin [16], which play roles in the pathogenesis of renal dysfunction [17, 18]. The renal tubule cells express specific receptors which mediate effects of individual cytokines [19, 20]. The expression patterns of cytokine profiles were different in acute kidney injury (AKI), glomerulonephritis (GMN), and end-stage kidney disease

(ESKD) [21, 22]. Previous reports have documented, the imbalance secretion of pro-inflammatory cytokines and their inhibitors was dysregulated in CKD patients [23].

Tumour necrosis factor alpha (TNF-α) is a potent immunomodulator and pro-inflammatory Th1 cytokine [24], and has been implicated in host defense mechanisms against intracellular bacteria [25, 26]. TNF-α and its receptors were involved in different inflammatory conditions leading to renal injury. Previous reports have documented the contrasting roles of TNF-α in proinflammatory and immunosuppressive activity in experimental models of lupus nephritis, [27, 28] and anti-TNF-α treatments also shown promising results in glomerulonephritis.

The present study was examined the expression level of proinflammatory Th1 cytokines such as IFN-γ and TNF-α in CKD patients in relevant with various clinical parameters and its secondary complications associated with renal dysfunction.

## Material and methods

### Enrollment of samples

A total of 133 CKD patients (24 females with mean age: 48.41 ± 15.53 yrs; 109 males with mean age: 50.69 ± 15.29 yrs) and 149 healthy volunteers (69 females with mean age: 43.34 ± 13.11 yrs; 80 males with mean age: 40.06 ± 13.15 yrs) were enrolled. The CKD patients were recruited from private hospitals from Madurai. The present study was included all the CKD samples such as patients with dialysis, Non-Dialysis, post-transplant and medication from the age groups of 15-80 yrs. Patients with malignancies, autoimmune, inflammatory diseases, pregnant women, pediatric kidney failures, AIDS were excluded. The well-defined detailed structured questionnaire was maintained for each patient. Written informed consent was collected from all participants and the study was approved by the Institutional Ethical Committee.

### RNA extraction and cDNA synthesis

The total RNA extraction was extracted with the Trizol reagent (Invitrogen). The RNA pellet was solubilized in DEPC-treated water. Reverse transcription was performed with RNA samples shown by UV spectrophotometry to be free of protein and phenol.

**Quantitative Real-Time PCR (RT-qPCR).**

Cytokine mRNA expression was performed on a Rotor-Gene Q cycler, by real time PCR (Qiagen) method. cDNA was diluted 1:10 with water and 20µl were used for amplification. qRT-qPCR was performed by denaturation and polymerase activation (95°C for 10 min), amplification of RT-qPCR products in a 45-cycle one-step PCR including denaturation (95°C, 10 s)/annealing (68°C–58°C, −0.5°C/cycle)/extension (72°C, 16 s) for each circle. The results of negative control samples were set as baseline level. Specificity of the amplification products was verified by melting curve analysis combined with agarose gel electrophoresis.

To determine the Ct value for TNF-α and IFN-γ, were normalized to the Ct values of β-actin as an endogenous control to yield ΔCt data. The relative expression for each sample was calculated using the 2-ΔΔCt formula as described previously.

**Statistical analysis**

Data were expressed as mean ± SEM. The data were analyzed with GraphPad Prism version 6.0 (GraphPad Software, Inc., San Diego, CA, USA.). Pearson's correlation coefficient was used to find the correlation between eGFR and Creatinine with p value of ≤0.05 as significant. Mann–Whitney U-test was used for testing the differences between the groups. Participant eGFR was calculated based on the CKD Epidemiology Collaboration (CKD-EPI) formula (Levey et al., 2009).

**Results**

**Demographical Characteristics.**

The demographic and biochemical profiles of 133 CKD patient and 149 control groups were shown in Table 1. The mean age average for CKD patients and controls were 49.41 ± 15.53 and 41.58±13.11 yrs respectively. Out of 133 CKD patients, 109 males (mean age average 50.69±15.29) and 24 females (mean age average 48.76 ± 15.56) were enrolled. There were more males than females, reflecting the well-known higher incidence of CKD in males. A total of 90.90% (n=120) patients had underwent dialysis and remaining 9.09% (n=12) patients had Non-Dialysis group. The most common documented comorbidities were diabetes + hypertension

(19.17% (n=51), followed by hypertension (15.78%; (n=42), and diabetes (3.83%; n=09), cardiomyopathy (0.37%; (n=01) and stroke (0.37%; (n=01).

Figure 1 depicts the laboratory values obtained upon hospital admission. The clinical parameters such as serum creatinine (7.99±3.45 mg/dl (range: 1.8-40.1 mg/dl) urea level (114.75 ±35.97 mg/dl (range: 35-243 mg/dl), and hemoglobulin (8.92±1.74 g/dl (range:5.2-13.6 g/dl) were observed for present study cohorts. The estimated glomerular filtration rate (eGFR) was 11.15±11.93 ml/min/1.73m$^2$). A significant negative correlation between Creatinine and eGFR were observed for dialysis (r= -0.78; p<0.0001) and Non-Dialysis group (r= -0.91; p<0.0001) (Figure 2). The disease severity progression showed that, 84.2% (n=112) of individuals fall under <15 eGFR (Figure 3). The most prevalent blood group among CKD patients was $O^+$ (22.34%) followed by $B^+$ (15.15%), $A^+$ (6.43%) and $AB^+$ (3.38%).

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**TNF-α and IFN-γ Expression Levels in CKD patients and controls**

We determined the mRNA levels of proinflammatory cytokines, TNF-α and IFN-γ in 133 CKD patients and 149 control subjects. Table 2 represents the fold change expression of TNF-α and IFN-γ in CKD patients and its complications. The upregulated expression for IFN-γ (FC: 1.76 ±0.36) and TNF-α (FC: 2.57±0.86) (p<0.004) in pooled CKD patients (Figure 4A). When we compared the cytokines expression levels between dialysis group of patients with controls subjects found that the upregulated mRNA levels for TNF-α (FC:2.60±0.88) and IFN- γ (FC:1.71±0.37) (p<0.0001) (Figure 4B). However, in Non-Dialysis group, a significant upregulation for IFN- γ (FC:1.60±0.86; p<0.0001) and down regulation for TNF-α (FC:0.19±0.13; p<0.0001) were observed (Figure 4C).

Further, the data were stratified based on CKD patients with diabetic complications, we found a significant upregulated expression for TNF- α (FC:1.79 ±0.63; p< 0.0001) and IFN- γ (FC:2.02 ±0.67; p< 0.001) (Figure 4D). Similarly, we found a significant upregulation were noticed for IFN-γ (FC:1.79 ±0.46; p<0.0001) and TNF- α (FC:1.87 ±0.51; p< 0.0001) in non-diabetic CKD patients

(Figure 4E). When we grouped the data based on with and without complications, we found a significant upregulated expression for IFN-γ (FC:1.64±0.51; $p<0.0001$) and down regulated expression for TNF- α (FC:0.65±0.26; $p<0.0001$) were observed in without complications (Figure 4F). A significant upregulated expression were observed for IFN-γ (1.82±0.39; $p<0.0001$) and TNF- α (2.27±0.67; $p<0.0001$) in with complications (Figure 4G)

**Correlation between TNF-α and IFN-γ expression in CKD patients**

Figure 5 represented a significant positive correlation between TNF-α and IFN-γ ($R=0.620$; $p< 0.0001$) in CKD patients when compared to controls.

**Discussion**

The mechanisms of renal function decline are not fully understood. It may be due to the abnormalities in several signaling pathways such as renin–angiotensin system, reactive oxygen species, endoplasmic reticulum stress, and proinflammatory cytokines [29]. Ischemic kidney injury triggers production of several cytokines, such as TGF-β, IFN-γ, IL-6, TNF-α, and IL-1β in the inflamed kidney [30, 31, 31]. Chronic inflammation is the major factor of morbidity and mortality in dialysis patients. The evaluation of cytokine release in dialysis patients is complex by the fact that the dialysis procedure per se seems to further stimulate cytokine production [32].

CKD is defined as a progressive loss of renal function, measured by a decline in glomerular filtration rate (GFR < 60 mL/min/1.73 m2) [13, 33], which is typically associated with irreversible pathological changes within the kidney. It has been documented that plasma and urinary markers are the early progressive in renal decline for T2DM [34]. The GFR can be estimated from serum concentrations of endogenous creatinine or cystatin C [35]. In our study, there was negative correlation between Creatinine and eGFR in dialysis and Non-Dialysis groups. The disease severity progression analysis sates that, 84.2% (n=112) of individuals fall under <15 eGFR (i.e.,very severe risk). The calculation of eGFR is the early diagnosis marker for CKD patients. In general, The Glomerular filtration rate is normally higher in early diabetes and patients with this symptom randomly. Our data are in concordance with the previous data showed that '$O^+$' blood group was the most prevalent in CKD patients followed by '$B^+$' blood groups [36].

The increased cytokine production has been described in ESKD patients, while markedly different cytokine concentrations were reported [37]. The proinflammatory cytokines TNF-α is a central importance in T and B-cell for their initiation of the immune response. Our study demonstrates the range of 2.27-2.60 fold increased TNF-α expression in CKD (pooled), dialysis and with secondary complications group of patients. The elevated circulating TNF-α levels in the ESRD patient may include factors, such as insulin resistance and obesity. Interestingly, down regulated expression for TNF-α were seen in Non-Dialysis and without secondary complications groups. Whereas in diabetes groups, 1.7 fold up-regulated expression for TNF-α was recorded. The decline of renal function may be associated with a significant increase in TNF-a activity in uremia [38]. The correlation between renal function and TNF-α and its soluble receptors was varied in varying degrees of renal failure [39]. Previous reports have shown to increase TNF-α synthesis and gene expression in patients with idiopathic nephrotic syndrome and focal glomerular sclerosis [40, 41]. TNF-α and IL-6 cytokines are in important for both acute and chronic inflammation are associated with CVD morbidity and mortality in the general population [42] and in predialysis and dialysis patients [43, 44]. The total effect of TNF-α is equilibrium between its proinflammatory and immunosuppressive function, which is determined by the cellular microenvironment and differs between the early and late phases of inflammation.

The disrupted gene expressions for IFN-γ, IFN-γR1, or IFN-γR2 display severe defects in IFN-γ mediated responses in host defense [45, 46]. IFN-γ affects both early and late events in organ transplants [47, 48] and to regulate a variety of renal diseases including autoimmune systemic lupus erythematosus in mice [49, 50] but not anti-glomerular basement membrane crescentic nephritis [51]. Our present data reported that, the IFN-γ expressions were observed in the range of 1.60-1.79 fold in CKD (pooled), dialysis, with and without secondary complications and non-diabetes. However, the 2.02 fold increased expressions for IFN- γ was noticed in diabetes groups. It has been reported in renal transplant model that lower IFN-γ gene expression causes less infiltration of leukocytes and allograft rejection, demonstrating that this cytokine is associated with inflammation process and kidney injury [52].

Excessive proinflammatory responses can lead to uncontrolled tissue damage altered the immune response in uremia causes inflammation in CKD patients [53, 54]. The limitation of our study is

the sample size and larger different cohort studies are recommended to derive a better understanding of cytokine network mediated inflammation in CKD patients. Thus, our data allow us to speculate that CKD patients have altered proinflammatory cytokine profile different from healthy control subject. In the future, targeting this proinflammatory cytokine network may provide clear mechanisms which mediate cytokine inflammation will lead to novel therapies to CKD.

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**Conflicts of Interest**

The authors declare that they have no conflicts of interest.

**Authors Contributions**

Designing the work: VS, RC and BK

Sample Collection and performed experiments: VS, SP and SKK

Result analysis and Interpretations: VS, RC

Manuscript Writing: RC, VS and BK

**Consent to Participate**

Written Informed Consent was obtained from all the volunteer participated in the study.

**Consent to Publish**

Informed consent was obtained from the Institution and volunteers to publish the outcome of the study and not any personal details.

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# Figures

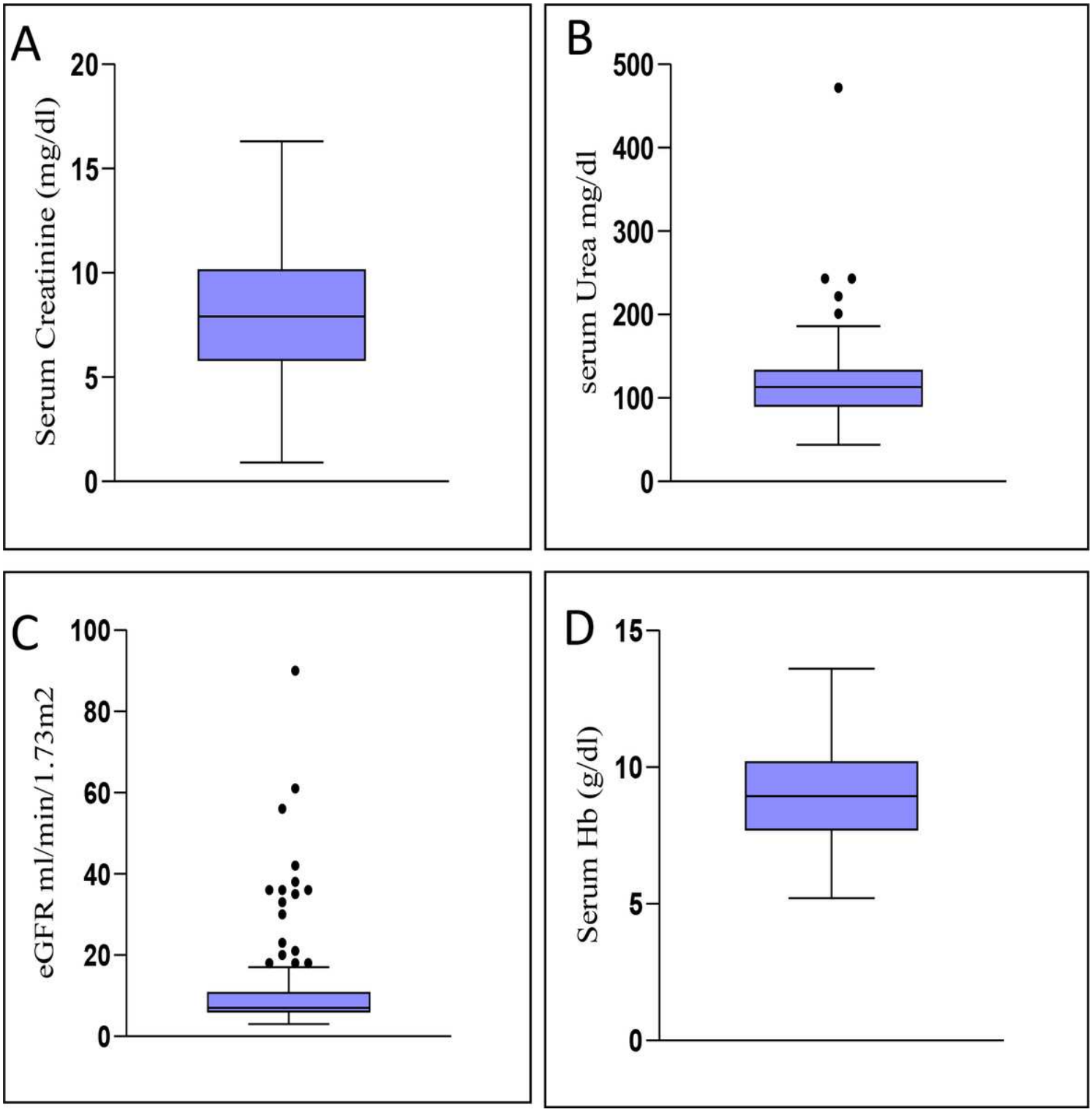



**Figure 1**

Clinical parameters of CKD patients based on laboratory results (A) Serum Creatinine (B) Serum Urea (C) eGFR(ml/min/1.72m2) (D) Serum HB.

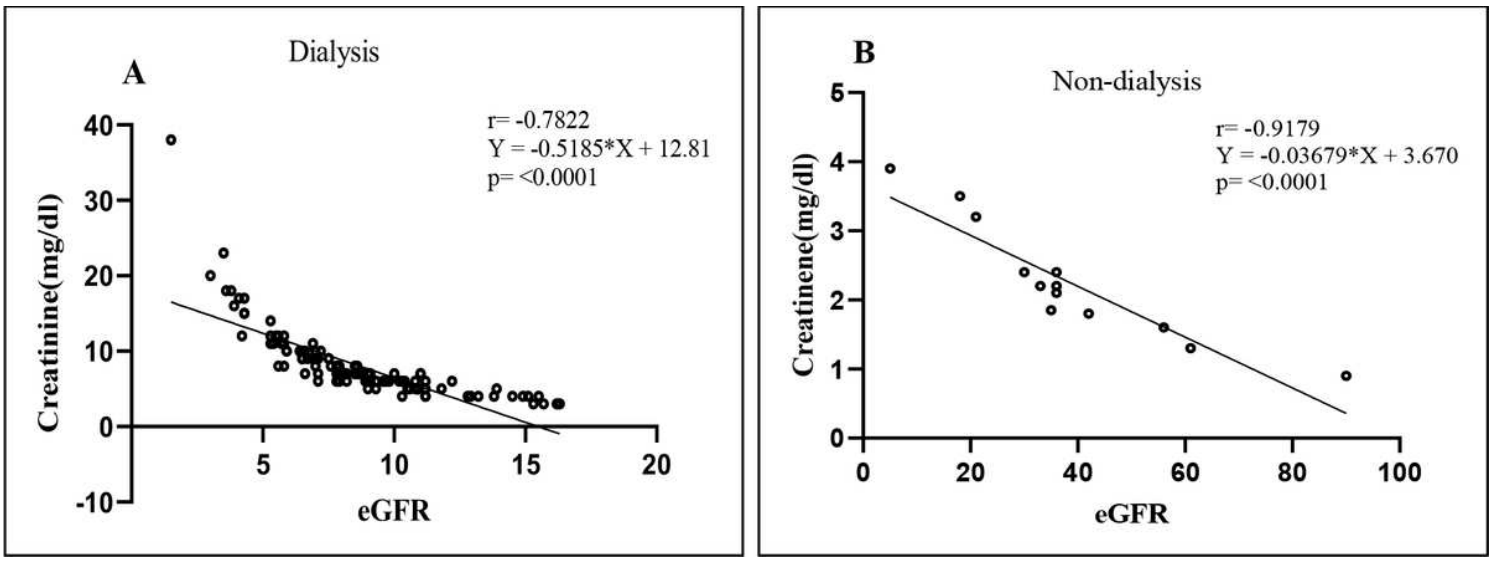



Figure 2

Correlation between Creatinine and eGFR (A)Dialysis (B) Non- Dialysis.

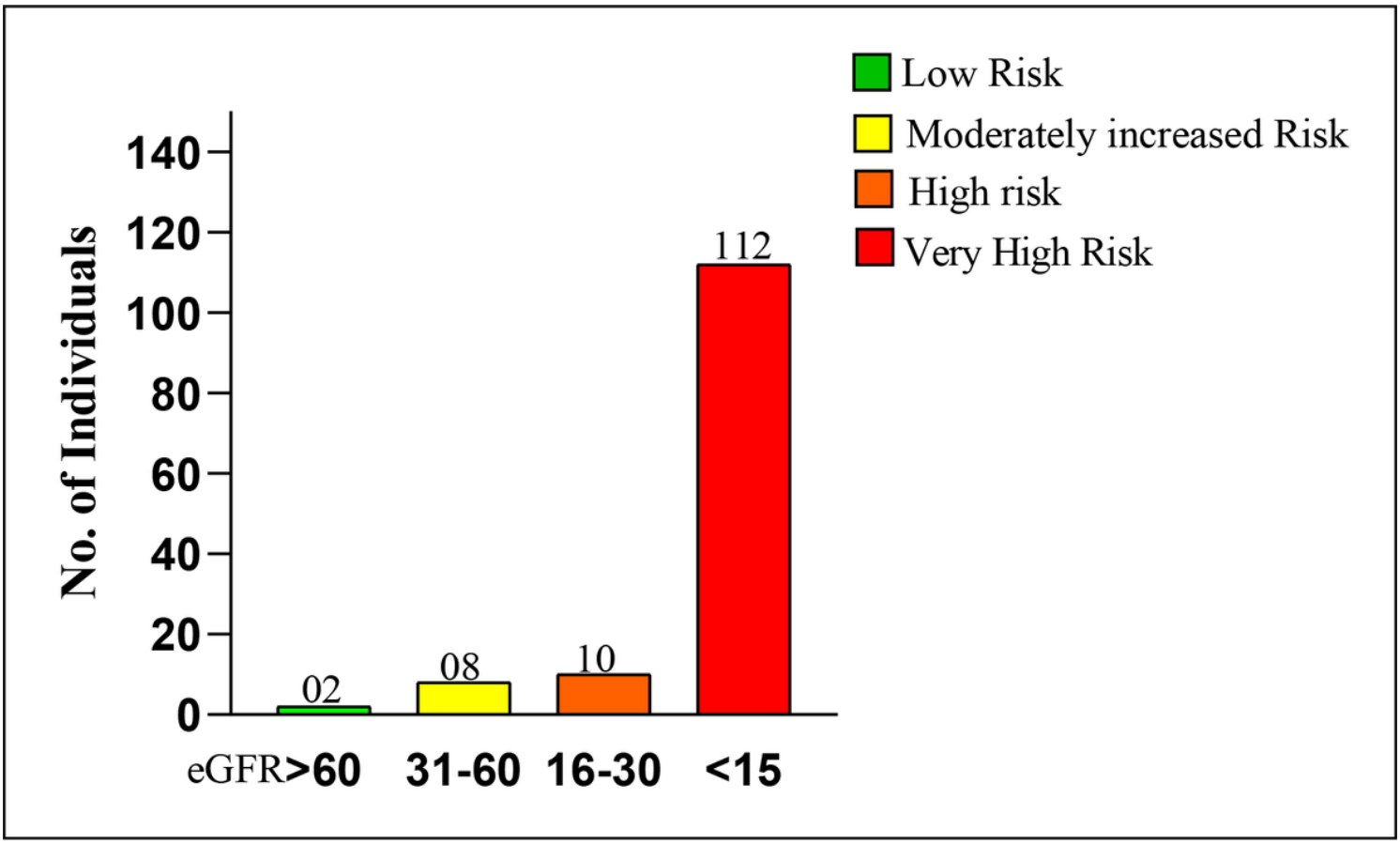
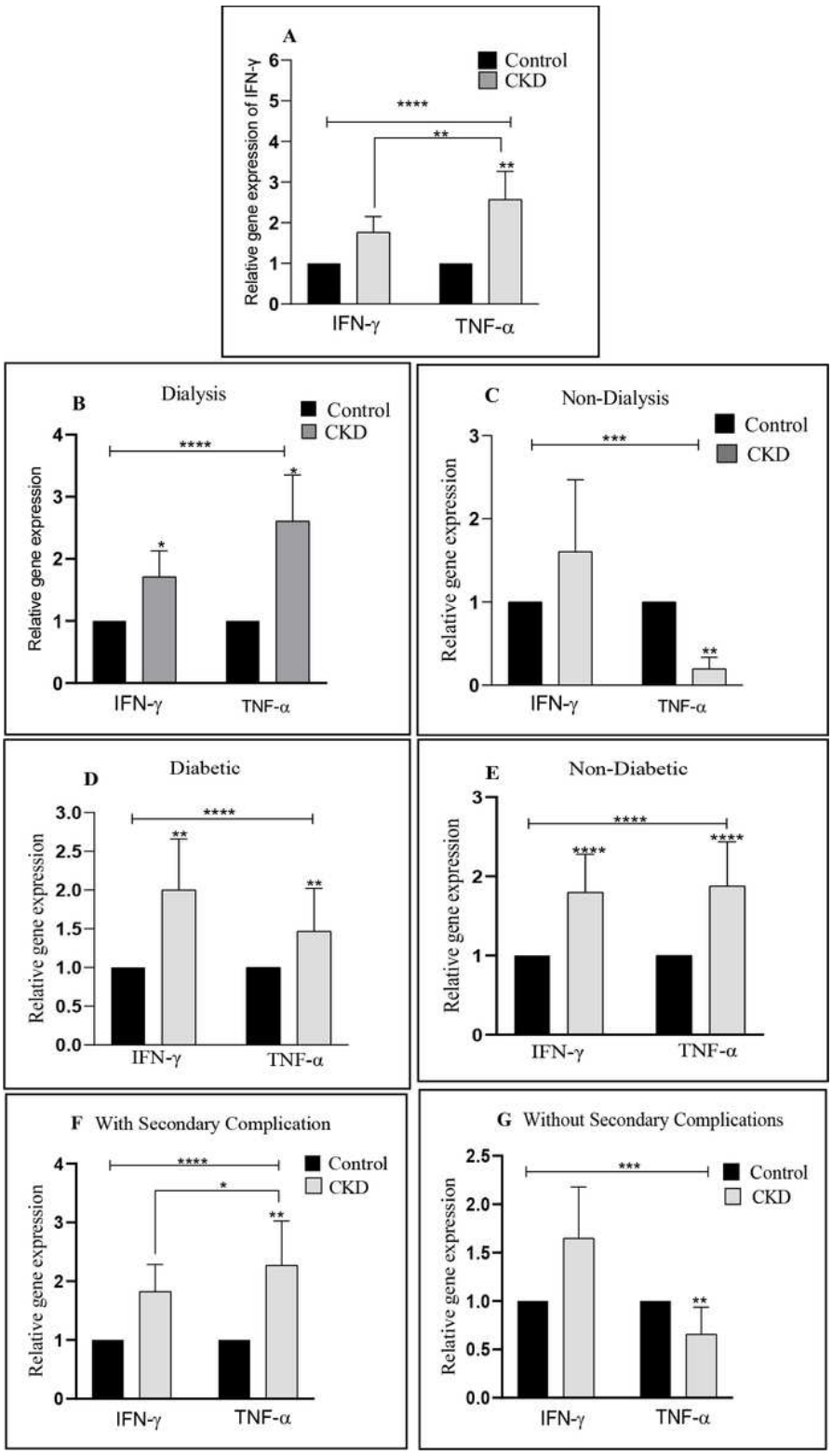



Figure 3

Severity of the disease progression with eGFR(ml/min/1.73m2)




## Figure 4

Relative Gene Expression of IFN-γ and TNF-α in CKD Patient and Control. (A) CKD(Pooled) (B) Dialysis (C) Non-Dialysis (D) Diabetic (E) Non-Diabetic (F) With Secondary complication (G) without Secondary complication.

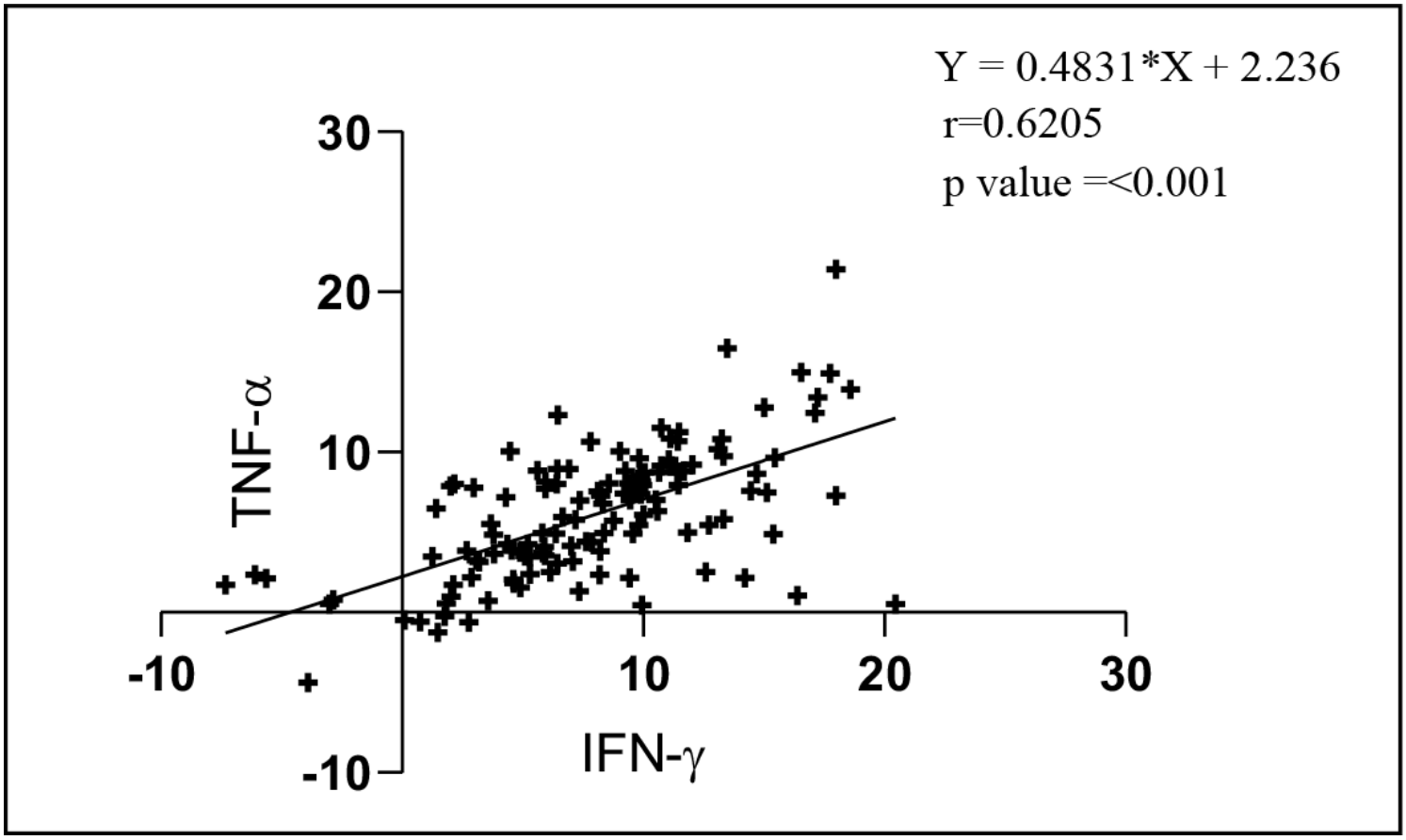



**Figure 5**

Correlation between TNF-α and IFN-γ among CKD patient with normalized Ct values

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Table1.pdf
- Table2.pdf