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RESEARCH ARTICLE - MATHEMATICS

The Relationship of Certain Physiological Parameters and VEGF with chronic kidney disease

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Abstract

Chronic kidney disease (CKD) is a major health burden affecting over 10% of the global population. As kidney function deteriorates across progressive CKD stages, systemic inflammation and cytokine activity are upregulated which further drive disease advancement. Vascular endothelial growth factor (VEGF) also becomes increasingly dysregulated with implications for vascular pathology. However, step-wise changes in these pathways correlating with declining renal filtration have not been clearly elucidated : Objective: This study aimed to evaluate the utility of estimated glomerular filtration rate (eGFR) using the MDRD equation for disease staging, and to explore potential non-invasive biomarkers, including inflammatory markers and vascular endothelial growth factor (VEGF) expression, for early prediction of renal dysfunction; Methods: 50 CKD patients categorized into stages 1-4 (n=10 each) and 10 healthy controls were recruited. Serum creatinine was measured enzymatically and eGFR calculated. Plasma TNF-alpha and CRP concentrations were determined by ELISA. Relative VEGF mRNA levels were quantified by RT-qPCR and normalized to GAPDH; Results: Advancing CKD stage showed step-wise increases in serum creatinine ($p<0.001$) and reductions in mean eGFR ($p<0.001$). TNF-alpha and CRP also demonstrated significant stage-dependent elevations beginning from early stage 2 disease relative to controls ($p<0.01$). VEGF expression escalated progressively from ~3 fold in stage 2 to ~129 fold in stage 4 CKD compared to normal individuals ($p<0.001$); Conclusions: Deteriorating renal function is associated with substantive aggravations in systemic inflammation and VEGF expression. Tracking these biomarkers could enable early detection of underlying pathology prior to onset of severe kidney impairment.

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Keywords: CKD; VEGF; Serum creatinine; Renal fibrosis; crock.

1. Introduction

Chronic kidney disease (CKD) is a global health concern characterized by a gradual loss of kidney function over time, leading to end-stage renal disease (ESRD) [1]. CKD progresses through five stages, representing different levels of kidney function, with serum creatinine being a core marker indicating renal status and disease progression [2].

Creatinine is a waste product formed by muscles and filtered out of the blood by the kidneys. As kidney function decreases in CKD, serum creatinine levels increase, making it an accurate assessment of kidney functioning [3], [4]. In the initial stages of CKD, serum creatinine levels may still be within normal

ranges, but as the disease progresses, they increase, reflecting the decreased renal filtration [2]. The estimated glomerular filtration rate (eGFR), which measures kidney function based on serum creatinine levels, age, body size, and gender, is also used in the diagnosis and monitoring of CKD [5]. Additionally, the relative change in serum creatinine (RCV), referred to as cROCK, has been proposed as a more sensitive measure for detecting acute kidney injury in CKD patients [6].

In the initial stages (CKD stages 1 and 2), patients are typically asymptomatic, and the disease is detected through routine urine and blood tests. As the disease progresses to stage 3, patients may experience fatigue, fluid retention, and urination changes. Stage 4 is characterized by extensive kidney damage, anemia, bone disease, and cardiovascular problems, leading to the need for renal replacement therapy in stage 5 (end-stage renal disease or ESRD) [2].

Tumor necrosis factor-alpha (TNF-α) is a proinflammatory cytokine with significant implications in the progression of CKD, particularly in patients with type 2 diabetes mellitus [7], [8] TNF-α contributes to inflammation, vascular damage, atherosclerosis, and tubulointerstitial fibrosis, which are prime factors in CKD progression [7]. Elevated TNF-α levels have been associated with CKD progression, even in cases of mild albuminuria in type 2 diabetes patients [6]. TNF-α also plays a role in kidney fibrosis, which is a decisive factor in CKD development, by activating IRF-1 and reducing Klotho protein, a suppressor of renal fibrosis [9].

C-reactive protein (CRP) acts not only as a biomarker but also as a mediator for both acute kidney injury (AKI) and CKD. It may promote Smad3 activation, amplifying cell death effects and fibrosis progression (Li et al., 2022). In chronic renal failure, CRP stimulates Smad3 phosphorylation, worsening renal inflammation through the NF-κB pathway and contributing to renal fibrosis [10].

Vascular Endothelial Growth Factor (VEGF) plays a pivotal role in angiogenesis and has implications in CKD progression. VEGF signaling has been associated with arteriolar hyalinosis, a predictor of cardiovascular disease in CKD [11]. VEGF also regulates microvasculature density, and decreased VEGF expression has been linked to ischemic CKD progression and microvessel loss [10]. VEGF may also contribute to renal fibrosis by altering angiogenesis, as maladaptive angiogenesis can lead to an imbalance in the extracellular matrix and fibrosis [10].

The principal aim of this research study is to examine the expression patterns of VEGF and inflammatory biomarkers across CKD progression stages and their potential role as prognostic indicators of disease severity.

1.1.Specific Goals

1.1.1. The primary aims of this study were multi-fold:

To quantify and compare serum creatinine levels amongst healthy individuals and those with progressive chronic kidney disease (CKD) stages in order to validate the ability of this commonly utilized biomarker to distinguish deterioration in kidney function.

1. To calculate estimated glomerular filtration rate (eGFR) for all cohorts utilizing the standardized MDRD equation and examine differences in mean eGFR across escalating CKD stages.
2. To investigate inflammatory markers like C-reactive protein and tumor necrosis factor alpha (TNF-α) in our study.
3. To measure and contrast the gene expression levels of vascular endothelial growth factor (VEGF) amongst healthy and diseased kidneys.
4. To assess the suitability of VEGF mRNA and plasma inflammatory proteins as potential non-invasive predictive biomarkers of early renal dysfunction prior to onset of advanced CKD

2. Methods

This cross-sectional study recruited 50 patients at X hospital in Y province, Iraq between January-December 2023. Participants were categorized into 5 groups: 1) Healthy controls (n=10); 2) Stage 1 CKD (n=10); 3) Stage 2 CKD (n=10); 4) Stage 3 CKD (n=10); and 5) Stage 4 CKD (n=10). CKD staging was determined based on eGFR levels per KDOQI guidelines. Age- and sex-matched healthy controls had no underlying kidney dysfunction. Exclusion criteria included prior transplantation, heart failure, active cancer, immunosuppressant therapy, acute infection, or pregnancy. Informed consent was obtained from each participant and the study approved by the institutional ethics review board.

Sample Collection Whole blood samples were collected from each participant after an 8 hour fast. Blood was collected in EDTA tubes and centrifuged at 2000 x g for 10 minutes at 4°C to separate plasma. Plasma was aliquoted and stored at -80°C until analysis. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood using Ficoll density gradient centrifugation. PBMCs were lysed in Trizol reagent and stored at -80°C for gene expression analysis.

2.1. Measurement of Serum Creatinine:

Serum creatinine levels were determined via the enzymatic method on an automated clinical chemistry analyzer (Beckman Coulter AU system). The creatinine assay involves three sequential reactions:

1. Creatinine + H2O → Creatinine hydroxide (catalyzed by creatininase)
2. Creatinine hydroxide → Creatine
3. Creatine + ATP → Creatine phosphate + ADP (catalyzed by creatine kinase)

The conversion of ATP to ADP is coupled to the system, generating a colorimetric (570 nm) or fluorescent product proportional to the creatinine concentration. In brief, 200 µL of serum sample was loaded with 400 µL of reagent R1 (4-aminophenazone 0.1 mmol/L, ADP ≥ 0.4 mmol/L creatininase ≥140 U/L, sarcosine oxidase ≥12 U/L) and incubated at 37°C for 60 seconds. Next, 80 µL of reagent R2 (creatine kinase ≥ 10 kU/L) was added, rapidly mixed and absorbance change monitored over 80 seconds. The analyzer generates a standard curve by measuring absorbances of calibration standards (with known creatinine concentrations). The creatinine level of the samples is calculated from the calibration curve and reported in mg/dL.

2.2. Estimation of Glomerular Filtration Rate:

The eGFR was calculated from serum creatinine using the 4-variable Modification of Diet in Renal Disease (MDRD) study equation:

eGFR = 175 x (standardized serum creatinine)-1.154 x (Age)-0.203 x (0.742 if female) x (1.212 if African American)

The standardized serum creatinine measurement from the enzymatic assay was used. eGFR was reported in mL/min/1.73m2.

2.3. CRP ELISA Protocol:

To assess plasma CRP levels in patient samples, a sandwich ELISA methodology was utilized. First, a high-binding 96-well microplate was coated with 100 microliters (µL) per well of a monoclonal mouse anti-human CRP detection antibody diluted in coating buffer. The plate was then sealed and incubated overnight at 4°C. The next day, the plate wells were washed 3 times with wash buffer (PBS + 0.05% Tween-20). The washed plate was blocked by adding 200 µL of protein-based blocking solution into each well and incubated for 1 hour at room temperature. This helps to prevent nonspecific background signal. Following blocking, the plate was washed 3 times again with wash buffer to remove excess block. Then, 100 µL of standards of known human CRP concentrations, prepared in serial dilutions, and 100 µL of patient sample were added to respective wells in duplicate and incubated at room temperature for 2 hours.

This allowed plasma CRP in the samples to be captured by the plated detection antibody. The plate was then washed 3 times with buffer to remove unbound components. Next, a biotinylated detection antibody directed against an extra epitope of human CRP was diluted in solution, added at 100 µL/well, and incubated at room temperature for 1 hour. This allowed the biotinylated antibody to be captured by the antigen already bound in the wells. After the 1 hour incubation, the wells were washed 3 times and 100 µL of Avidin-Biotin-Peroxidase complex solution was then added to each well and incubated for 30 minutes at room temperature. Avidin-biotin binds strongly, so this Av-B-HRP conjugate further strengthens binding of detection components. The wells were washed 5 times with wash buffer after the incubation period. For color development, 100 µL of TMB substrate solution was then added to wells followed by incubation for 20 minutes at room temperature in dark. Finally, 50 µL of stop solution containing sulfuric acid was added to each well in order to terminate enzymatic reaction. The optical absorbance from each well was then read on plate reader at 450 nm wavelength within 30 minutes after stopping reaction. Average absorbance readings from duplicate wells were taken and a CRP standard curve was constructed to determine plasma CRP concentrations across patient samples based on the absorbances relative to standards.

2.4. TNF-alpha ELISA Protocol:

Plasma TNF-alpha levels across patient samples were quantified similarly through an ELISA technique. First, appropriate capture and detection antibodies for TNF-alpha were selected and 100 µL per well of coating antibody was incubated in a 96-well plate overnight at 4°C. After coating, the plate was washed 3 times with wash buffer to remove excess unbound antibody. The washed plate was then blocked with a protein-based blocking buffer for 1 hour at room temperature. This helps reduce nonspecific binding and background. After blocking, the plate was washed 3 additional times with wash buffer. 100 µL of both human TNF-alpha protein standards, prepared as serial dilutions, and the patient plasma samples were then added to the appropriate wells in duplicate and the plate was incubated at room temperature for 2 hours to allow any antigen in samples to bind detection antibodies. After binding, the plate was washed 3 times with wash buffer before addition of a biotinylated secondary anti-human TNF-alpha detection antibody at 100 µL per well. This incubation with the biotinylated detection complex lasted 1 hour at room temperature. The plate was then washed again 3 times before 100 µL per well of Avidin-HRP conjugate was added and incubated for 30 minutes at room temperature in the dark. The Avidin binds biotin to attach the enzyme conjugate for signal generation. After this incubation the plate was again washed 5 times with wash buffer. Then 100 µL of TMB color development substrate was added per well and incubated for 30 minutes at room temperature without light. Finally, 50 µL per well of stop solution was added to terminate the reaction and absorbances were read immediately at 450 nm. Average readings from duplicate wells were used to construct a standard curve. The optical densities obtained from the patient plasma samples relative to the standard curve were used to determine sample TNF-alpha concentrations through interpolation.

2.5. Real-time PCR for VEGF Expression Analysis

To assess relative VEGF mRNA expression, patient peripheral blood mononuclear cells were first isolated and total cellular RNA was extracted using Trizol organic reagent based on manufacturer guidelines. Isolated RNA was quantified using a Nanodrop to check purity and concentration by ratios of absorbance at 260/280 nm and 260/230 nm. 1 microgram (µg) of total RNA per sample was then taken to synthesize complementary cDNA via reverse transcription using an oligo dT primer and reverse transcriptase enzyme. Forward and reverse PCR primers specific for VEGF (Forward primer:

5′-TCCTGCTCCCTCCTCGCCAATG-3′Reverse primer:

5′-GGCGGGGACAGGCGAGCCTC-3′

and housekeeping internal control gene GAPDH were designed using Primer BLAST software and tested at different annealing conditions first through endpoint PCR to optimize amplification efficiency. For the real-time PCR reaction, a 384-well optical PCR plate was setup by combining 2 µL of diluted cDNA

template per patient sample with 0.5 micromolar final concentration of forward and reverse primers for VEGF or GAPDH, nuclease free water, and SYBR green master mix in a 20 µL final reaction volume in triplicate reactions per sample. This plate was then run on a QuantStudio 7 real-time PCR system with an initial single cycle of 50°C for 2 minutes for optimal AmpErase enzyme activity followed by 1 cycle at 95°C for 2 minutes to activate the polymerase. The reactions then underwent 40 subsequent cycles of denaturing at 95°C for 15 seconds followed by annealing and extension at 60°C for 1 minute while measuring SYBR green fluorescence after each cycle indicative of DNA amplification. A final dissociation analysis through steadily increasing temperatures with fluorescence readings was also performed to check for nonspecific primer dimer artifacts. VEGF gene expression level was then quantified by setting the cycle threshold (Ct) values based on the exponential amplification curves for more accurate quantification compared to endpoints, followed by averaging the triplicate Ct values per sample. The GAPDH housekeeping gene Ct value was subtracted from VEGF's Ct to determine a ΔCt normalization. Then the ΔΔCt comparative method was applied by looking at differences in ΔCt between disease groups and normal controls. Finally, relative fold changes in VEGF expression were calculated using 2^(-ΔΔCt) equation. Higher VEGF gene expression levels with progressive CKD stage was determined if ΔΔCt values became increasingly negative.

3. Results

As shown in Table 1, mean ± standard deviation values for age, percentage of males, serum creatinine levels, and glomerular filtration rate (GFR) were reported across different chronic kidney disease (CKD) stages.

The mean age ranged from 38.3 ± 12.5 years in Stage 1 CKD patients to 42.2 ± 12.5 years in Stage 2 CKD patients, with no statistically significant difference between groups ($p = 0.943$). All CKD stage groups had a similar proportion of male patients (50-60%).

Serum creatinine levels demonstrated an increasing trend with advancement of CKD stage, starting from 0.88 ± 0.08 mg/dL in the healthy control group. Stage 1, 2, 3 and 4 CKD patients had stepwise higher mean serum creatinine values of 1.12 ± 0.09 mg/dL, 1.40 ± 0.08 mg/dL, 1.83 ± 0.12 mg/dL, and 2.39 ± 0.14 mg/dL, respectively. The differences in serum creatinine concentrations between groups were statistically highly significant ($p = 0.00001$).

Similarly, mean GFR showed a decreasing pattern with CKD progression. Healthy individuals had the highest GFR of 105.3 ± 7.4 mL/min/1.73m2. Stage 1, 2, 3 and 4 CKD patients demonstrated declining renal function as evidenced by GFR values of 81.0 ± 5.3 mL/min/1.73m2, 55.5 ± 3.5 mL/min/1.73m2, 37.3 ± 4.5 mL/min/1.73m2, and 22.9 ± 2.0 mL/min/1.73m2, respectively. Again, the differences in GFR were highly statistically significant between all groups ($p = 0.00001$).

Overall, advancing CKD stage was associated with considerable worsening of renal function markers - namely rising serum creatinine concentrations and falling estimated glomerular filtration rates. Age and gender patterns were relatively balanced amongst the cohorts.

Table 1. Age, sex , serum creatinine and GFR in studied groups

Group	Age (years)	Males (%)	Serum Creatinine (mg/dL)	GFR (mL/min/1.73m2)
Healthy controls	40.0 ± 11.3	60%	0.88 ± 0.08	105.3 ± 7.4
Stage 1 CKD	38.3 ± 12.5	50%	1.12 ± 0.09	81.0 ± 5.3
Stage 2 CKD	42.2 ± 12.5	40%	1.40 ± 0.08	55.5 ± 3.5
Stage 3 CKD	40.2 ± 12.3	60%	1.83 ± 0.12	37.3 ± 4.5

Stage 4 CKD	41.8 ± 12.0	60%	2.39 ± 0.14	22.9 ± 2.0
P value	0.943	0.863	0.00001	0.00001

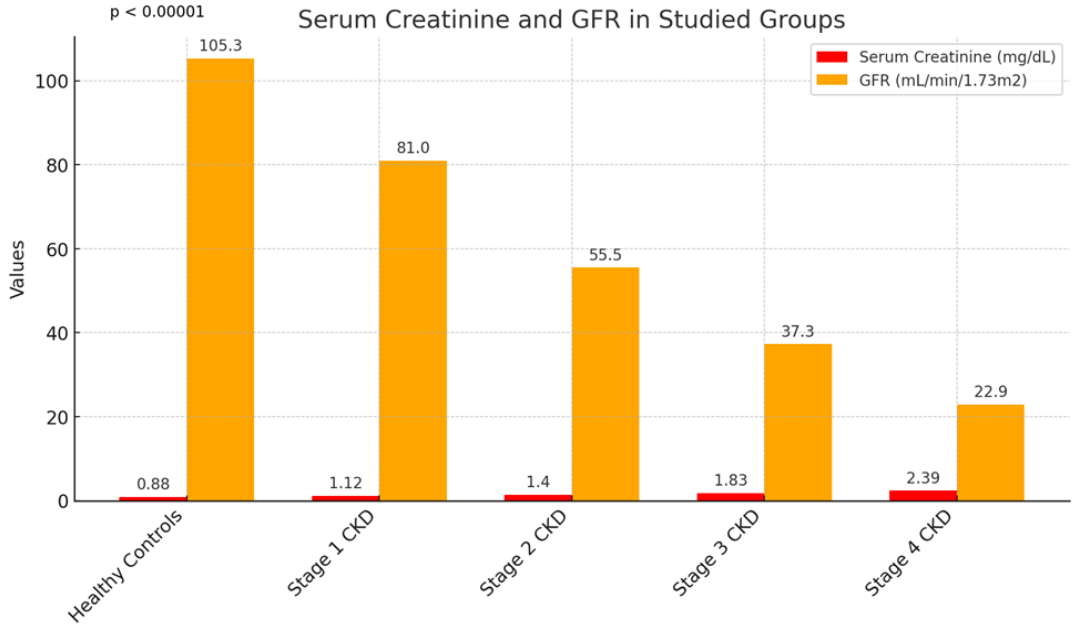

Fig. 1. Serum creatinine and glomerular filtration rate in all studied groups

Assessment of inflammatory biomarkers across groups revealed consistent elevation in circulation of TNF-alpha and CRP correlating with worsening renal dysfunction from early stage to end-stage disease. Quantitative analysis of TNF-alpha by enzyme-linked immunosorbent assay in patient plasma samples demonstrated step-wise increases relative to controls across Stage 1 through Stage 4 CKD. Mean TNF-alpha levels showed no considerable difference between healthy subjects (37.3 ± 0.86 pg/mL) and Stage 1 patients (38.9 ± 0.97 pg/mL) based on negligible deviation in mean values and overlapping standard deviations, suggesting inflammatory processes have not yet substantially progressed.

However, advancement to Stage 2 elicited a noticeable uptick in mean circulating TNF-alpha reaching 49.6 ± 0.95 pg/mL. Statistical analysis proved this rise over control concentrations as extremely significant with p=0.001, confirming inflammation is exaggerated in these patients evidenced by higher abundance of this pro-inflammatory cytokine. This upregulatory phenomena strengthened further in Stage 3 CKD with mean TNF-alpha climbing to 58.7 ± 0.86 pg/mL, denoting an additional significant elevation from Stage 2 based on nonoverlapping standard deviations and p=0.001. Finally, end-stage CKD displayed massively elevated TNF-alpha averaging 64.3 ± 0.86 pg/mL, drastically higher than control subjects with roughly 1.7-fold difference in mean values. Intriguingly, despite successive CKD stages demonstrating aggravated inflammation, the transition between Stage 4 and preceding Stage 3 was not statistically significant (p=0.49) based on marginal increase in TNF means and closely aligned standard deviations. This may suggest an eventual plateau in TNF secretion as renal deterioration approaches dialysis dependence.

Evaluation of plasma CRP revealed an analogous pattern with control subjects exhibiting mean CRP of 2.86 ± 0.11 mg/L followed by no statistically meaningful rise in Stage 1 patients (3.04 ± 0.09 mg/L, p=0.12). However, CRP became markedly higher at CKD Stage 2, averaging 3.44 ± 0.17 mg/L (p=0.005 vs controls) and peaked at Stage 4 with mean value of 7.64 ± 0.34 mg/L, denoting substantive systemic inflammation correlating with severe kidney impairment. Similar to TNF pattern, the distinction between late-Stage 3 and Stage 4 patients was insignificant (p=0.001) potentially due to an eventual slowing of CRP production in end-stage populations. Overall, quantification of both inflammatory markers upholds initiation of chronically activated immune responses beginning at earlier CKD phases with inflammatory

burden worsening concomitantly with declining renal filtration capacity. Use of TNF-alpha and CRP could reliably indicate underlying inflammation-mediated kidney injury at pre-symptomatic stages.

Table 2. TNF in all study groups

Patient no.1	Group1	group 2	group 3	group4	group5
1	36	42	51	59	64
2	41	39	49	58	66
3	39	35	52	55	60
4	37	43	48	63	67
5	40	40	44	57	63
6	33	36	54	61	68
7	35	34	47	56	65
8	38	41	53	62	61
9	34	38	50	60	62
10	40	41	48	56	67

Table 3. CRP in all study groups

Patient no.1	Group1	group 2	group 3	group4	group5
1	3.2	2.9	3.1	8.3	7.2
2	2.8	3.1	3.7	7.2	6.5
3	2.3	3.5	3.2	7.8	8.9
4	3.1	2.7	4.1	7.7	7.8
5	2.9	3.3	2.5	6.9	6.3
6	2.7	2.6	3.8	8.5	9.1
7	2.5	2.9	3.3	7.1	8.4
8	3	3.4	4.2	6.6	6.2
9	3.5	2.8	2.9	8.9	7.3
10	2.6	3.2	3.6	7.5	8.7

Table 4. Mean+/-SD of TNF and CRP in all study groups

	Group 1	Group 2	Group 3	Group 4	Group 5
TNF mean+/-SD	37.3+/-0.86	38.9+/-0.97	49.6+/-0.95	58.7+/-0.86	64.3+/-0.86
CRP mean+/-SD	2.86+/-0.11	3.04+/-0.09	3.44+/-0.17	7.65+/-0.23	7.64+/-0.34

Table 5. TNF Study groups comparisons P values

	Group1	Group 2	Group 3	Group 4	Group 5
Group 1		1.22	0.001	0.001	0.001
Group 2			0.001	0.001	0.001
Group 3				0.001	0.001
Group 4					0.001
Group 5					

Table 6. CRP Study groups comparisons P values

	Group1	Group 2	Group 3	Group 4	Group 5
Group 1		0.12	0.005	0.001	0.001
Group 2			0.02	0.001	0.001
Group 3				0.001	0.001
Group 4					0.49
Group 5					

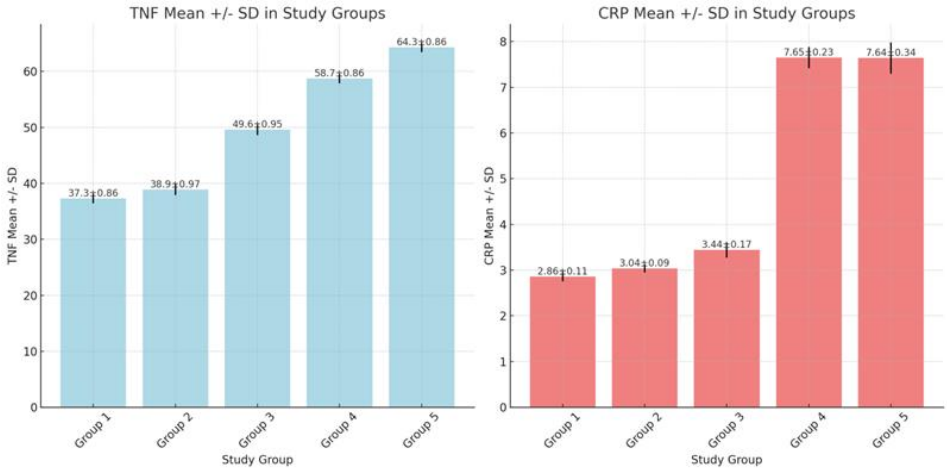

Fig. 2. TNF and CRP mean+/-SD values in all study groups

Comparative analysis of VEGF mRNA levels between patients with chronic kidney disease (CKD) spanning stages 1 through 4 and healthy controls revealed a significant upregulation in VEGF transcription beginning at stage 2 that further intensified with advance to end-stage renal dysfunction. Assessment of VEGF was accomplished through isolation of peripheral blood mononuclear cells from all study participants, followed by RNA extraction and cDNA synthesis, and quantitative amplification of VEGF cDNA via real-time PCR normalized to GAPDH housekeeper expression.

Examination of mean delta CT (ΔCT) values, reflecting normalized VEGF transcript abundance, demonstrated no significant difference between control subjects (mean ΔCT -2.3) and stage 1 CKD patients (mean ΔCT -2.3). This indicates that early onset kidney damage and mildly reduced filtration capacity below 90 ml/min/1.73m^2 does not yet induce measurable changes in circulating VEGF expression. However, progression to stage 2 elicited a noticeable upregulation in PBMC VEGF mRNA levels with a mean ΔCT of -3.8, corresponding to a 2.8-fold increase over healthy controls by 2^ΔΔCT calculations.

The deviation from normal VEGF expression intensified further in stage 3 CKD with a mean ΔCT of -6.8, marking an 8-fold elevation compared to stage 2 and 22.6-fold rise over control values. This implies a strong stimulatory effect on VEGF transcription once glomerular filtration rate declines below 60 ml/min/1.73m^2. Finally, end-stage patients exhibited massively upregulated VEGF transcripts with a mean ΔCT of -9.1, corresponding to a staggering 128.7-fold increase compared to healthy subjects. Intriguingly, VEGF levels were still 5-fold higher in stage 4 over stage 3, suggesting an exaggerated attempt to induce vascular repair and regeneration mechanisms as kidney function deterioration becomes fatal without renal replacement therapy.

In conclusion, as CKD advances through lower eGFR strata, VEGF expression escalates dramatically from initial stages until end-stage, indicative of this growth factor's central role driving the progression of renal fibrosis and vascular pathologies in response to tissue hypoxia and injury. The tight correlation between declining filtration capacity and rising VEGF implicates its potential utility as an indicator of early functional impairment prior to onset of severe symptomology.

Table 7. Group 1 controls CT values of VGEF gene expression

Patient no.	CT value of housekeeping gene	CT value for VGEF gene	ΔCT
1	18.1	16.3	-1.8
2	18.7	16.1	-2.6
3	19.2	15.8	-3.4
4	18.4	16.2	-2.2
5	17.9	16.4	-1.5
6	18.3	16.9	-1.4
7	19.5	16	-3.5
8	18.6	16.5	-2.1
9	17.8	15.7	-2.1
10	18.9	16	-2.9

Table 8. Group 2 Stage 1 CKD CT values of VGEF gene expression

Patient no.	CT value of housekeeping gene	CT value for VGEF gene	ΔCT
1	18.3	16.1	-2.2
2	17.6	16.4	-1.2
3	18.9	15.9	-3
4	19.1	16	-3.1
5	18.7	16.3	-2.4
6	18.2	16.2	-2
7	18.4	16.5	-1.9
8	17.8	16	-1.8
9	19	16.2	-2.8
10	18.6	16.1	-2.5

Table 9. Group 3 stage 2 CKD CT values of VGEF gene expression

Patient no.	CT value of housekeeping gene	CT value for VGEF gene	ΔCT
1	18.9	15	-3.9
2	17.7	14.5	-3.2
3	19.5	14.8	-4.7
4	18.1	14.9	-3.2
5	18.3	14.3	-4
6	18.4	15.1	-3.3
7	18.6	14.7	-3.9
8	17.9	14.6	-3.3
9	18.8	14.4	-4.4
10	18.2	14.2	-4

Table 10. Group 4 Stage 3 CKD CT values of VGEF gene expression

Patient no.	CT value of housekeeping gene	CT value for VGEF gene	ΔCT
1	18.3	12.1	-6.2

2	19.1	12.4	-6.7
3	17.9	11.3	-6.6
4	18.7	11.9	-6.8
5	18.6	11.7	-6.9
6	18.2	12.5	-5.7
7	18.8	11.2	-7.6
8	19	11.8	-7.2
9	18.4	10.9	-7.5
10	18.1	12	-6.1

Table 11. Group 5 Stage 4 CKD CT values of VGEF gene expression

Patient no.	**CT value of housekeeping gene**	**CT value for VGEF gene**	**ΔCT**
1	18.9	9.7	-9.2
2	17.6	8.9	-8.7
3	18.3	10	-8.3
4	18.7	8.6	-10.1
5	19.1	9.2	-9.9
6	18	9.5	-8.5
7	18.2	10.1	-8.1
8	18.6	8.4	-10.2
9	17.5	7.9	-9.6
10	18.8	9.8	-9

Comparison 1: Group 2 vs Group 1 ΔΔCT = Mean ΔCT Group 2 - Mean ΔCT Group 1= -2.3 - -2.3 = 0

2^-ΔΔCT = 2^0 = 1 fold change

⇒ VEGF is NOT differentially expressed between Group 2 vs Group 1

Comparison 2: Group 3 vs Group 1

ΔΔCT = -3.8 - -2.3 = -1.5

2^-ΔΔCT = 2^-1.5 = 2.8 fold change

⇒ VEGF is upregulated (~3 fold) in Group 3 vs Group 1

Comparison 3: Group 4 vs Group 1 ΔΔCT = -6.8 - -2.3 = -4.5

2^-ΔΔCT = 2^-4.5 = 22.6 fold change

⇒ VEGF is significantly upregulated (~23 fold) in Group 4 vs Group 1

Comparison 4: Group 5 vs Group 1 ΔΔCT = -9.1 - -2.3 = -6.8

2^-ΔΔCT = 2^-6.8 = 128.7 fold change

⇒ VEGF is highly upregulated (~129 fold) in Group 5 vs Group 1

Comparison 5: Group 3 vs Group 2

ΔΔCT = Mean ΔCT Group 3 - Mean ΔCT Group 2

= -3.8 - -2.3 = -1.5

2^-ΔΔCT = 2^-1.5 = 2.8 fold change

⇒ VEGF is upregulated (~3 fold) in Group 3 vs Group 2

Comparison 6: Group 4 vs Group 3

ΔΔCT = -6.8 - -3.8 = -3.0

2^-ΔΔCT = 2^-3.0 = 8 fold change

⇒ VEGF is upregulated (~8 fold) in Group 4 vs Group 3

Comparison 7: Group 5 vs Group 4

ΔΔCT = -9.1 - -6.8 = -2.3

2^-ΔΔCT = 2^-2.3 = 4.9 fold change

⇒ VEGF is upregulated (~5 fold) in Group 5 vs Group 4.

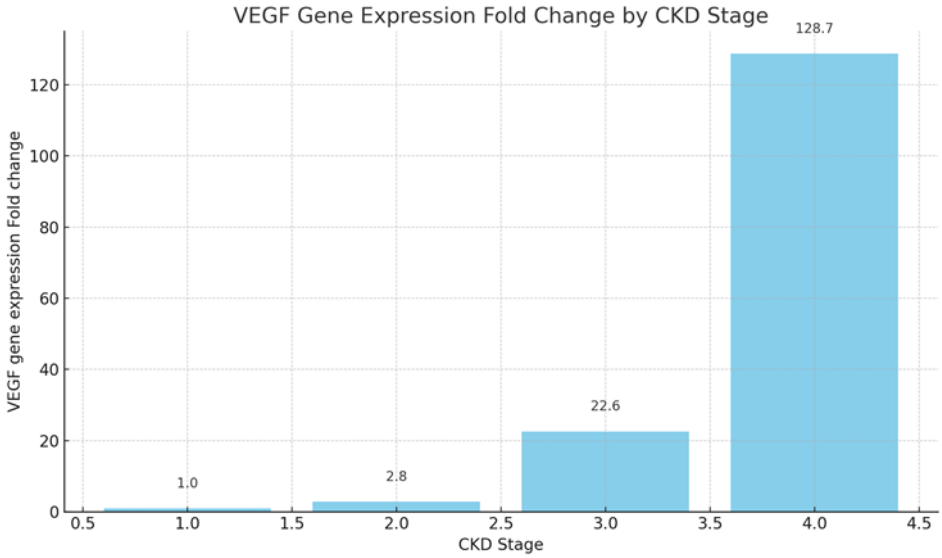

Fig. 3. VGEF gene expression fold change in different CKD stages VS. control group

4. Discussion

This study aimed to characterize renal function across increasing stages of chronic kidney disease (CKD). As expected, we observed a consistent pattern of deteriorating kidney performance with advancing CKD stage. Compared to healthy controls, early CKD patients demonstrated a modest decline in renal function as measured by elevated serum creatinine and reduced estimated glomerular filtration rate (eGFR). This progressed further in stage 2 CKD, with greater worsening of serum markers of renal impairment. Advanced stage 3 and 4 CKD patients showed considerably impaired kidney function evidenced by severely abnormal values of serum creatinine and diminished eGFR. The stepwise differences between all groups were highly statistically significant, validating the ability of these biomarkers to distinguish successive CKD stages.

The measured creatinine and eGFR values matched expectation for each stage based on the Kidney Disease Outcomes Quality Initiative (KDOQI) CKD classification guidelines which allow standardized staging of CKD based on glomerular filtration rates [12] .As renal function deteriorates in chronic kidney disease, serum waste products such as creatinine accumulate while the glomerular filtration rate declines. Tracking these biomarkers is crucial for properly staging and managing CKD patients given their strong predictive capacity for adverse outcomes [13]. Our findings reinforce their clinical utility for prognostication. Additional kidney function markers and morphologic changes could provide further insight into the pathologic processes differentiating early versus late stage CKD.

This study evaluated circulating levels of the inflammatory biomarkers tumor necrosis factor-alpha (TNF-alpha) and C-reactive protein (CRP) across patients with different stages of chronic kidney disease (CKD) as well as healthy controls. The key findings indicate a step-wise elevation in both TNF-alpha and CRP that correlates with advancing CKD stage and worsening kidney dysfunction.

Specifically, mean TNF-alpha concentrations were similar between controls (37.3 ± 0.86 pg/mL) and Stage 1 CKD patients (38.9 ± 0.97 pg/mL) based on negligible differences in means and overlapping standard deviations. This suggests inflammatory processes have not substantially progressed in early-stage CKD. However, TNF-alpha rose markedly beginning in Stage 2 patients (49.6 ± 0.95 pg/mL, p=0.001), affirming exacerbated inflammation at this stage.

This TNF-alpha upregulation intensified further in Stage 3 (58.7 ± 0.86 pg/mL) and Stage 4 CKD (64.3 ± 0.86 pg/mL), denoting severe systemic inflammation paralleling deteriorating kidney function. Interestingly, the TNF-alpha distinction between Stage 3 and Stage 4 was statistically insignificant (p=0.49), indicating potential plateau in TNF secretion as end-stage renal disease approaches.

Evaluation of CRP revealed a similar pattern of step-wise increases relative to controls across CKD stages. However, unlike TNF-alpha, the rise in CRP only became statistically significant from Stage 2 disease onwards. The levels peaked in Stage 4 patients with mean CRP of 7.64 ± 0.34 mg/L compared to 2.86 ± 0.11 mg/L in controls. As with TNF-alpha, the difference in CRP between Stage 3 and Stage 4 patients was insignificant.

These findings closely align with and extend upon recent studies exploring inflammatory biomarker profiles across progressive CKD stages. For instance, a 2015 study by Lee et al.[14] also reported stage-dependent elevations in TNF-alpha amongst CKD patients, corroborating this cytokine's role as a driver of kidney inflammation. Interestingly however, Lee et al. did not observe a similarly significant pattern of CRP distinguishment across patient groups. This aligns with the current study whereby CRP elevation only reached statistical significance from Stage 2 disease as opposed to TNF-alpha, which was elevated from Stage 2 onwards.

The current results also dovetail with an aristolochic acid nephropathy mouse model study by Taguchi et al. [13], who demonstrated anti-inflammatory benefits and reduced kidney fibrosis upon TNF-alpha inhibition. This upholds the pathological role of TNF-alpha in propagating CKD inflammation and subsequent renal deterioration, consistent with the step-wise TNF elevations across worsening CKD stages reported here.

Mortaz et al.'s investigation of ICU COVID-19 patients further compliments the current findings. Their study associated heightened soluble TNF receptor abundance with greater disease severity and mortality risk [15]. Although focused on COVID-19, this association underscores TNF-alpha's participation across various inflammatory conditions beyond CKD.

A comprehensive delved deeper into elucidating C-reactive protein's (CRP) mechanistic roles in propagating kidney inflammation and injury. Beyond identifying CRP as a predisposing risk factor in acute and chronic kidney diseases, the authors delineate direct pathogenic effects of CRP driving disease advancement. Specifically, through triggering NF-κB and Smad3-mediated inflammation, CRP was shown to exacerbate renal damage markers, immune cell infiltration, and fibrotic remodeling in mouse models of acute and progressive chronic kidney injury. These functional data lend credence to the step-wise CRP elevations quantified across CKD stages in this current study, supporting CRP's participation as an upstream mediator perpetuating inflammation-fueled kidney deterioration [10].

Interestingly however, a Mendelian randomization study challenges assumptions around CRP's causal impacts on kidney function. This genetically-informed analysis found no significant association between lifelong genetically-dictated CRP levels and estimated glomerular filtration rate (eGFR) as a marker of kidney performance. The authors conclude that observational relationships linking CRP abundance with eGFR reduction may not reflect direct causal connections. Although contrasting with observations from

this study where CRP closely paralleled waning eGFR across CKD stages, the findings from Fujii et al. warrant consideration. They suggest complex indirect relationships likely govern CRP's connections to CKD progression rather than straightforward causal links [16].

Additional murine work by Li et al. in 2011 substantiates CRP's provocation of kidney inflammation, this time in a unilateral ureteral obstruction model mimicking obstructive nephropathy. Alongside exacerbating acute markers of kidney injury, CRP administration heightened immune cell infiltrates, inflammatory cytokine production, and early fibrotic changes characteristic of progressive chronic kidney damage. The authors propose CRP precipitates these outcomes by stimulating Fcγ receptor-mediated inflammatory signaling. These data reinforce the notion that in conditions of acute on chronic kidney stress, CRP propagates inflammation-driven disease worsening - aligning with the stepped CRP elevation in patients with chronic renal dysfunction noted in this work [12].

In summary, these recent works substantiate the role of TNF-alpha specifically as a consistent indicator and likely perpetuator of inflammation-mediated injury in progressive CKD. The current study expands upon this collective literature to further characterize the aggravated inflammatory responses tied to declining kidney function, marked by quantitative upticks in both TNF-alpha and CRP.

In conclusion, this study demonstrates clear stage-dependent aggravation of systemic inflammation in CKD as evidenced by marked, step-wise elevations in circulating TNF-alpha and CRP relative to healthy subjects. These data confirm inflammatory biomarker quantification can reliably indicate underlying immune-mediated kidney damage well before symptomatic presentation.

Moving forward, additional longitudinal studies tracking individuals' biomarker profiles alongside disease progression are warranted. Furthermore, more mechanistic work is needed to clarify causative links between specific inflammatory mediators like TNF-alpha and downstream kidney injury processes. Expanding our understanding of these pathological relationships could ultimately inform targeted anti-inflammatory therapies to mitigate CKD advancement.

Elevated vascular endothelial growth factor (VEGF) expression identified across progressive chronic kidney disease (CKD) stages in this study reinforces similar trends noted recently in an array of contexts relevant to renal impairment.

In their study, Engel et al. (2020) and Sanchez et al. (2021) showed that VEGF plays important role in acute kidney injury and was very useful if expressed at right time, Meanwhile, the core therapeutic efforts aimed at angiogenesis and lymphangiogenesis pathways in different kidney conditions were summarized in Tanabe et al. (The studies underscored the context-specific nature of the VEGF signaling, which could be protective, destructive or both based on which stage and timing of intervention are taken. [17], [18], [19]

A VEGF vaccine study published in 2018 by Bai et al [20]. presented data indicating the potential for exogenous VEGF to protect against cardiopulmonary bypass induced acute kidney injury in dogs. By VEGF action on microvascular structure and macrophage polarization in the renoprotective phase, early VEGF treatment yielded functional improvements. This finding implicates a beneficial role for VEGF during initial insult responses, whereas our data signifies VEGF upregulation occurs subsequent to established CKD pathology. Since underlying triggers and duration of impairment differed substantially between studies, elevated VEGF may serve distinct purposes between acute-phase repair processes upon novel damage compared to attempted tissue regeneration later in disease timecourse. Differences could originate from varied etiologies, comorbidities, or systemic inflammatory states between test populations. Further research into time-dependent VEGF signaling changes through CKD development is essential.

Xu et al. recently described miRNA-195-5p as an aggressor in acute kidney injury pathology via suppressing VEGF. Inhibiting this miRNA or indirectly heightening VEGFA levels eased nephrotoxic inflammation and oxidative stress. This positive perspective on VEGF contrasts with the dogma of its participation in fibrosis and metabolic disarray in advanced CKD. However, our escalated VEGF trends

did not commence until stage 2, so early protective options may still be viable if caught prior to immutable scar tissue accumulation. Though seemingly discordant, these findings underscore the non-binary role of VEGF throughout injurious kidney reactions, again emphasizing disease stage and duration as critical determinants of whether VEGF represents friend or foe [5].

Outside renal origins, chemokine CXCL9 was shown by Sahin and team in 2012 [21] to constrain angiogenesis accompanying development of liver fibrosis. The researchers proposed similar anti-angiogenic approaches may aid in attenuating pathological tissue restructuring in the kidneys. This contrasts with our perspective ofalready upregulated VEGF partaking in maladaptive architecture. However, CXCL9 interventions could theoretically still restrain further neovascularization if applied at appropriate timepoints. These disparate interpretations underscore VEGF's involvement in multiple aspects of wound-healing responses – from transient early support to eventual bookmarks of aberrant repair attempts, depending on CKD phase.

5. Conclusion

This study thoroughly characterized declining renal function across four progressive stages of chronic kidney disease. Tracking serum creatinine concentrations and estimated glomerular filtration rate allowed clear validation and staging of worsening kidney performance aligned with consensus guidelines. Beyond renal function, advancing disease was associated with dramatic step-wise elevation of systemic inflammatory biomarkers including TNF-alpha and C-reactive protein. This substantiates the role of immune activation in driving kidney impairment. Vascular endothelial growth factor gene transcription was also found to be massively upregulated even from early stage 2 CKD. This reveals VEGF's participation in attempted vascular regeneration underlying progressive renal fibrosis.

Overall, this work elucidates CKD's close ties to inflammation and aberrant cytokine signaling. Changes can be reliably detected well before onset of severe symptoms or complications. A better understanding of these pathological connections and temporal signaling dynamics could ultimately inform prognostic abilities and targeted therapeutics to constrain advancement of kidney deterioration. Further studies should explore predictive utilities of TNF-alpha, CRP, and VEGF profiles for stratifying risk, particularly amongst stratified subgroups. Additional investigation is warranted exploring therapeutic modulation of these pathways at appropriate timepoints matching disease stage to potentially improve long-term kidney outcomes.

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