ORIGINAL ARTICLE

Deciphering the Expression of *Klotho, Superoxide Dismutase 1, Catalase, p53*, and *Cyclophilin* Genes in Pathogenesis of Chronic Kidney Disease

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ABSTRACT

OBJECTIVE: To analyze the expression of genes involved in OS scavenging, defense activation and to understand their contribution to CKD pathogenesis.

METHODOLOGY: A case-control study was conducted among 150 participants in Ibn-e-Sina Hospital, Multan, from January to August 2023. The relative expression of antioxidants, including Klotho (KL), Catalase (CAT), Cyclophilin A (CyPA), tumor protein p53 (p53), and Superoxide dismutase 1 (SOD1), was quantified using gene-specific primers and real-time qPCR. Data were analyzed by one-way analysis of variance (ANOVA) via GraphPad Prism. The correlation among the *KL, CAT,* and *SOD1* genes was evaluated via matrix scatter plots.

RESULTS: The results show that the relative expression of KL and CAT genes was significantly downregulated in acute kidney injury (AKI) and CKD patients, whereas SOD1 was upregulated in AKI and downregulated in CKD patients compared with controls. The levels of *p53* were upregulated by >3-fold in CKD and AKI patients. *CyPA* was upregulated by >1.5-fold in patients with CKD and AKI. Among CKD stage-wise distribution, the levels of KL and CAT genes were downregulated, while SOD1 was unaffected. The levels of *p53* were >9-fold, 6-fold, and >3-fold upregulated in CKD stage 5 to 3, respectively. *CyPA* was >1.8-fold upregulated in stages 5 and 4 versus control. The *KL* gene was positively correlated with the *CAT* gene.

CONCLUSION: Differential expression levels of *KL, CAT, SOD1, p53*, and *CyPA* genes suggest their potential role in mediating OS scavenging, regulation, pathogenesis, and progression of kidney disease.

KEYWORDS: CKD, AKI, Kidney, Disease, Pathogenesis.

INTRODUCTION

Chronic kidney disease (CKD) has become a significant health issue that impacts >10% of the global population[1]. Acute kidney injury (AKI) is defined as a sudden reduction in kidney function, whereas CKD is a steady loss of kidney function over time[2,3]. An estimated glomerular filtration rate (eGFR) <60 mL/min/1.73 m² for 3 months indicates CKD, characterized by tubular atrophy, glomerulosclerosis, vascular rarefaction, arteriosclerosis, and nephron loss. The activation of fibrotic and inflammatory pathways, mitochondrial dysfunction, genetics, and cellular senescence leads to CKD.[2] High reactive oxygen species (ROS) lead to oxidative stress (OS), which lowers the activity of antioxidative enzymes. This imbalance leads to the oxidation of proteins, nucleic acids, carbohydrates, and lipids, resulting in cell apoptosis and necrosis[4]. Increased ROS in early kidney diseases causes tissue damage via dysregulation linked to AKI and CKD[5]. Approximately 90% of cellular ROS is produced by mitochondria. OS occurs when ROS production exceeds removal, affecting multiple cellular components. The kidney, being highly active, is vulnerable to OS-induced damage that can result in CKD[6]. Defense systems depend on micronutrient intake and antioxidant enzyme production, such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX)[7]. CAT, mainly located in the proximal tubule cytoplasm, is crucial for combating OS by regulating ROS, specifically by decomposing hydrogen peroxide (H_2O_2) into water (HO_2) and oxygen (O_2), thus maintaining redox balance in kidney function[7,8]. Superoxide dismutase (SOD) is another antioxidant that protects cells from OS by converting superoxide radicals into H_2O_2 and O_2, which are subsequently reduced to water by catalase or GPX. SOD is highly expressed in the kidneys and inhibits the oxidative inactivation of nitric oxide (NO)[7,8]. SODs comprise three isoforms (SOD1, SOD2, SOD3) in the kidney. SOD1 is the most prevalent SOD; its depletion increases NF-κB signaling and causes oxidative DNA damage in renal tissues[7]. Klotho (KL) is a transmembrane protein primarily found in healthy kidneys and recognized as an aging-suppressor protein. Its levels decline with age, contributing to kidney function loss and being linked to aging-related diseases, including hypertension, CKD, diabetes, cancer, and cardiovascular diseases[9]. *p53* is a tumor suppressor gene that plays roles in apoptosis, DNA damage repair, senescence, and growth detention[10]. *p53* levels remain low in normal cells due to its interactions with the ubiquitin ligase, which promotes p53 degradation[11]. Increased p53 expression is associated with CKD progression[12]. CyPA is a peptidyl-prolyl cis/trans isomerase-active immunophilin. It is a widely expressed cellular protein, particularly in proximal tubular epithelial cells of the kidney. Its expression and secretion changes are crucial for various physiological processes and the pathophysiology of kidney-related diseases[13].

The KL, CAT, SOD1, p53, and CyPA genes play important roles in kidney function, and their altered expression is associated with CKD pathogenesis. Therefore, the study aimed to analyze the expression of KL, CAT, SOD1, p53, and CyPA genes in CKD and AKI patients to investigate their roles in pathogenesis, thereby aiding early diagnosis and disease control.

METHODOLOGY

Study design

A case-control study was organized in Ibn-e-Sina Hospital in Multan, Pakistan, from January to August 2023. Informed consent was obtained from all participants in accordance with the Declaration of Helsinki. Patients with dialysis, specific immune therapy, uterine fibroid, cancer, steroid therapy, rheumatoid arthritis, systemic lupus erythematosus, acute infection, septic shock, hypotension, and COVID-19 were excluded from this study. In contrast, patients with diabetes, hypertension, cardiovascular disease, arthritis and glomerulonephritis were included.

Participant selection

150 participants were selected. CKD and AKI were defined according to the Kidney Disease: Improving Global Outcomes (KDIGO) criteria. CKD patients were further sub-grouped by stages of the disease via Modification of Diet in the Renal Disease (MDRD) equation as described by Yang W-C 2023[14]. 5ml of whole blood samples (biopsy) were collected from all participants by venipuncture into anticoagulant EDTA tubes and stored at -20°C in a refrigerator for further use.

RNA Extraction

RNA extraction was performed using the total RNA purification micro kit (Catalogue number 4373872). A 1.5% agarose gel was used to confirm the integrity of the extracted RNA via Bio-Rad agarose gel electrophoresis, and the quantity was measured using a Thermo Scientific NanoDrop 2000 spectrophotometer.

cDNA synthesis

The extracted RNA was converted to cDNA via the Thermo Scientific RevertAid First Strand cDNA Synthesis Kit (catalogue number K1622) and stored at -20°C for further use.

Primer designing

Klotho (KL), Catalase (CAT), Cyclophilin A (CyPA), *tumor protein p53 (p53)* and *Superoxide dismutase 1 (SOD1)* genes were selected for Real-time qRT-PCR. *Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)* was used as a housekeeping gene. The Primer3Plus web interface (https://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) was used for designing the primers. The genetic sequences of the selected genes were retrieved from NCBI (http://www.ncbi.nlm.nih.gov/nucleotide) and are listed in **Table I**. All the designed primers were then checked for homology and specificity using the BLAST bioinformatics tool (www.basic.northwestern.edu/biotools/oligocalc.html), and mismatches and secondary structure within the primers were avoided.

Table I: Forward and reverse primers to study the expression of selected genes and the housekeeping gene (*GAPDH*)

Gene	Primer	Sequence
KL	F	GCCCACATACTGGATGGTATCAA
	R	ACTGCACTCAGTACACACGGTGA
CAT	F	CGTGCTGAATGAGGAACAGA
	R	AGTCAGGGTGGACCTCAGTG
SOD1	F	GGCAAAGGTGGAAATGAAGA
	R	ACCACAAGCCAAACGACTTC
	F	TGCCATCGCCAAGGAGTAG
CyPA	R	TGCACAGACGGTCACTCAAA
p53	F	CCCCTCCATCCTTTCTTCTC
	R	ATGAGCCAGATCAGGGACTG
GAPDH	F	GGTGGTCTCCTCTGACTTCAACA
	R	ACCAGGAAATGAGCTTGACAAAG

Note: F= Forward Primer, R= Reverse Primer

Gene expression analysis through qRT-PCR

Amplification KIT qRT-PCR was performed to evaluate the expression of *KL, CAT, SOD1,* and *CyPA* via the 7500 Fast Real-Time PCR System. TaqMan Fast Universal PCR Master Mix was utilized, with each 25µL reaction containing 12.5µL Maxima SYBR Green/ROX q Master Mix (2X), 0.5µM of forward and reverse primers, and water. Finally, 0.5 µL of cDNA was added to the PCR plate for the RT-PCR program. The initial denaturation step was set at 94 ^{o}C for 5 minutes, followed by another 30sec denaturation at 94 ^{o}C. Annealing was allowed for 30 sec at 60 °C, and the extension step was at 72 °C for 30 sec. By normalizing to *GAPDH* expression, the relative quantification method $2^{-\Delta\Delta Ct}$ was used to assess the quantification of the target transcript. Each sample was quantified in triplicate.

Statistical Analysis

Data were displayed as mean ± standard deviation (SD) across patients in scatter plots using GraphPad Prism. GraphPad Prism was used to analyze the data using the Tukey multiple-comparison test and one-way analysis of variance (ANOVA). Asterisks (*) indicate rising levels of statistical significance ($p < 0.05$), whereas "ns" indicates non-significant data ($p > 0.05$). The associations among *the KL, CAT, and SOD1 genes were evaluated using Pearson correlation coefficients (r) and corresponding p-values, which were presented in* a matrix scatter plot.

RESULTS

Demographic parameters

The present study included a total of 150 participants: 108 with CKD, 15 with AKI, and 27 healthy controls, as shown in **Figure I**. Among 108 CKD patients, 9 were classified as CKD stage 2, while 12, 21, and 66 were categorized as CKD stage 3, 4, and 5, respectively, as shown in **Figure II**. The mean age of cases (kidney disease patients) was 45±15 years, respectively, while controls had a mean age of 42±15 years. In the cases, 59% were male, and 36% were female, while in the control group, 60% were male and 40% were female. The research highlights the demographic parameters within the studied groups.

Genetic expression among AKI and CKD

Oxidative stress plays a role in the development of atherosclerosis and the advancement of renal damage in patients with kidney disease. The expression levels of *the KL, CAT, SOD1, p53, and CyPA genes play a role in elucidating the pathogenesis of CKD and AKI*. The relative expression levels of *KL* and *CAT* were significantly ($p < 0.0001$) downregulated in CKD and AKI patients compared to controls, whereas the SOD1 gene was 1.5-fold upregulated in AKI patients and downregulated in CKD patients compared to controls, as shown in **Figure I**. The expression levels of *p53* were significantly ($p < 0.001$) upregulated by > 6-fold and > 3-fold in CKD patients and AKI patients, respectively, compared to controls **(Figure Ie).** The *CyPA* was significantly ($p < 0.0001$) upregulated by >1.5-fold and >2-fold in CKD and AKI patients, respectively, as shown in **Figure If**. Further, the relative expression of *the CAT, SOD1, and CyPA genes was higher in AKI patients than in* CKD patients. Overall, these results indicate downregulation of *KL*, *CAT*, and *SOD1*, and upregulation of p53 and CyPA, suggesting progressive kidney damage and an activated cellular stress response in kidney disease patients.

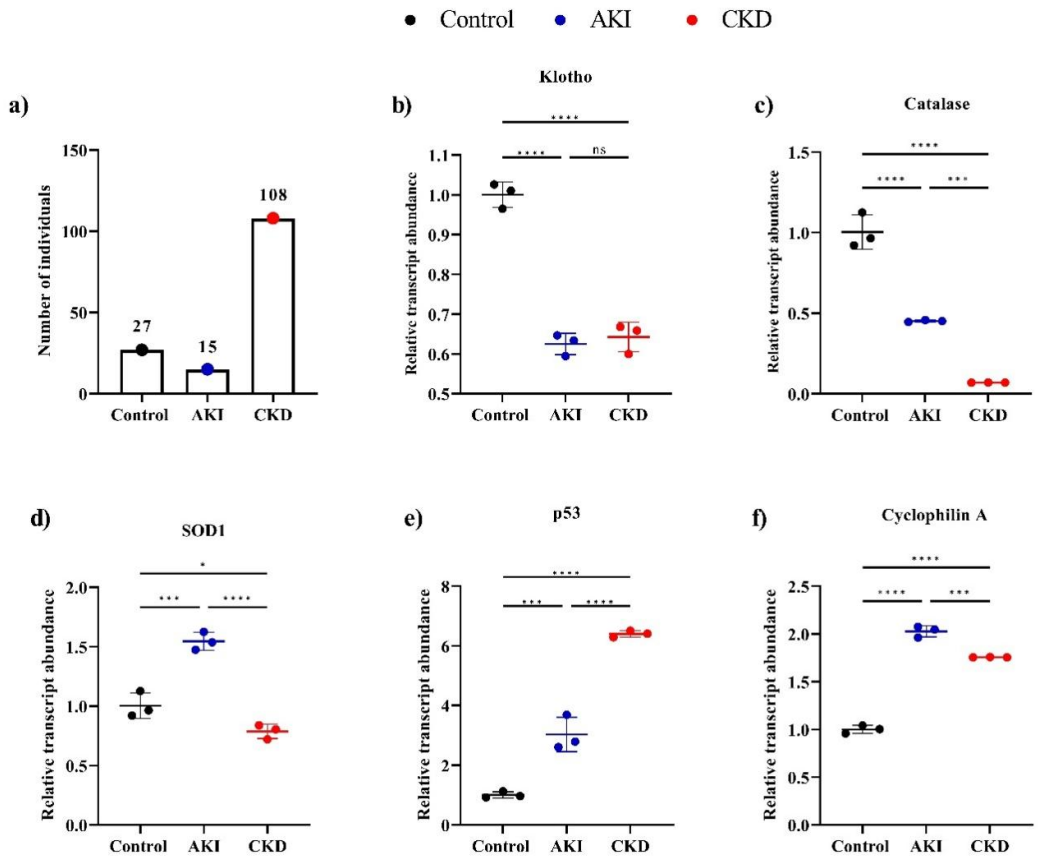

Figure I: Relative transcript abundance of *KL*, *CAT, SOD1*, *p53* and *CyPA* genes

The mean values were normalized to *GAPDH* =. SD is indicated by the error bar, and the line indicates the means of each group. Ns = non-significant, asterisks (*) = statistically significant at given probability level(s).

Genetic expression among CKD stages

As shown in **Figure I**, the expression levels of *the KL, CAT, SOD1, p53, and CyPA genes were disturbed; therefore, their levels were further investigated across* CKD stages. In a stage-wise distribution, the expression of the KL and CAT genes was downregulated in all CKD stages compared to controls, as shown in **Figure II**. The relative expression levels of the *SOD1* gene were found to be similar to those of the control (**Figure IIc)**. p53 levels of p53 were significantly upregulated (>9-fold, 6-fold, >3-fold, and >1-fold) across CKD stages (stage 5 to stage 2) compared to control, as shown in **Figure IIe**. The level of *CyPA* was significantly upregulated (>1.8-fold) in CKD stages 5 and 4 compared to the control. In contrast, it was of significantly different from control in CKD stages 3 and 2, as shown in **Figure IIf.** Overall, these results indicate that the downregulation of *KL, SOD1,* and *CAT,* along with upregulation of *p53* and *CyPA* in CKD stages, indicates reduced antioxidant defense and heightened cellular stress, peaking in stages 4 and 5.

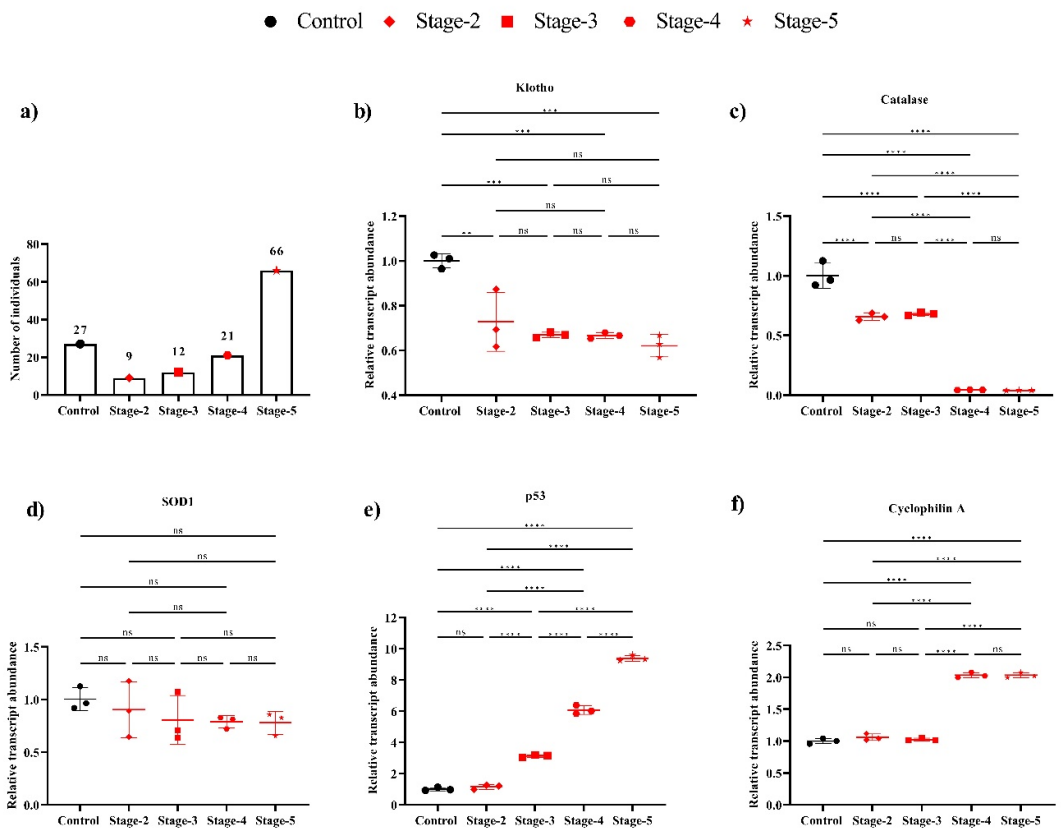

Figure II: Relative transcript abundance of *KL*, *CAT, SOD1*, *p53* and *CyPA* genes across CKD stages.

The mean values were normalized to *GAPDH*. SD is indicated by the error bar, and the line indicates the means of each group. Ns = non-significant, asterisks (*) = statistically significant at given probability level(s).

Correlation among KL, CAT, and SOD1 genes

The genetic correlation can help to understand the causality of relationships among genes. Therefore, the correlations of *KL, CAT, and SOD1* were examined to understand their relationship among them. The presented correlation **Table II** delves into the gene expression relationships within the control subgroup, focusing on *KL, CAT,* and *SOD1*. Correlation coefficients (r) are provided alongside p-values, revealing potential associations between these genetic expressions. Examining the correlations reveals several noteworthy patterns. **Klotho-F** exhibits a positive correlation with **Catalase** (r = 0.195), indicating limited associations as shown in **Figure III**. These correlations provide initial insights into potential connections between the examined gene expressions among cases. The significance of these correlations, as indicated by the p-values, varies across the pairs. The notable correlations could suggest shared regulatory mechanisms or potential interactions among these genetic expressions. Further research and analyses are required to elucidate the underlying biological significance of these correlations and their potential implications in the context of kidney health and disease. Among cases, Klotho showed a weak significant positive correlation with catalase. No significant correlation was seen between Klotho and *SOD-1*. Similarly, no significant correlation was observed between catalase and SOD-1 **(Figure III)**. Therefore, *the KL gene was positively correlated with the CAT gene, but SOD-1 showed no association with KL or CAT.*

Table II: Correlation of gene expression of *KL, CAT,* and *SOD1* among kidney disease patients

		KL	*CAT*	*SOD1*
KL	**r**	-	0.195	0.063
	p-value		0.026	0.478
CAT	**r**		-	0.159
	p-value			0.071
SOD1	**r**			-
	p-value			

Note: *r: Correlation coefficient,* ***n:*** *number*

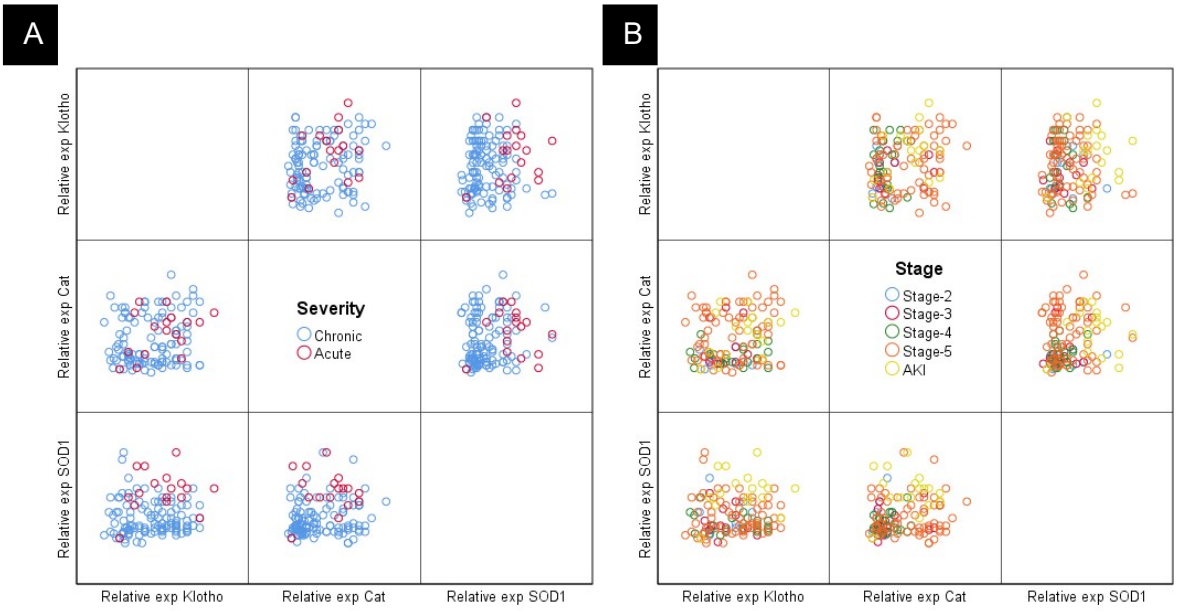

Figure III: Correlation of *KL, CAT,* and *SOD1* genes in relation to severity of disease among AKI and CKD (A) and CKD stages (B).

DISCUSSION

Chronic Kidney Disease (CKD) is a progressive disorder characterized by irreversible/permanent loss of kidney function, frequently associated with molecular and cellular alterations.[15] Understanding the underlying genetic mechanisms is critical for elucidating disease progression. In the present study, we investigated the differential expression of *KL*, *CAT, SOD1*, *p53,* and *CyPA* genes to understand the pathogenesis in kidney disease patients across CKD, AKI and control groups. The expression patterns may indicate oxidative burden and cellular compensatory responses in renal tissues.

Hassan MH et al. [15] reported lower Klotho levels in AKI and CKD patients than in controls, consistent with our study. Further, our study found lower expression in AKI versus CKD (**Figure Ib)**. Shimamura Y et al.[16] found that serum klotho levels were lower in patients with CKD stages 2 to 5 than in stage 1 patients, consistent with our study, which shows downregulation of the *KL* gene in CKD stages 3 to 5 versus stage 2 (**Figure IIb).** Devaraj S 2012[17] found elevated klotho levels in CKD patients, which is contrary to our study; this may be because CKD was identified as a creatinine level of more than 2 mg/dL over 12 months in their study, while in our study, CKD was identified by the MDRD equation according to KDGIO guidelines. The age of the patients was much older (54 years) than in our study. Age has a major impact on of levels, an anti-ageing protein[9]. Further, the sample size was small, which may limit the generalizability of their findings to the entire CKD population.

Sulaiman SH 2021[18] reported that OS has a strong influence on cellular and tissue damage in AKI and CKD patients and found significantly lower CAT activities in CKD and AKI patients compared to controls, findings that are also consistent with our study (**Figure Ic)**. Further, in our study, the *CAT* gene is downregulated in CKD and AKI patients, particularly in CKD stages 4 and 5 compared to stages 2 and 3 (**Figure IIc)**. Catalase protects kidneys from oxidative stress; loss of its activity leads to fibrosis and progressive renal injury[7,8.] Our study found the *KL* gene was positively correlated with the *CAT* gene (**Figure III)**. No contrary data were found. Pathare G 2020[19] reported lower levels of KL and CAT in hypertensive patients compared with controls. They reported a positive correlation between CAT and KL, which is somewhat consistent with our study of kidney disease participants.

SOD1 gene downregulation in CKD patients and upregulation in AKI patients were observed in our study (**Figure Id)**.

Sulaiman SH 2021[18] found significantly lower SOD activities in CKD and reported that oxidative stress has a strong influence on cellular damage and tissue in AKI and CKD patients, which is consistent with our study. Scholze A et al.[20] reported significantly higher expression of the SOD1 gene in CKD patients compared to controls, which is contrary to our findings. An increase in *SOD1* gene expression might be due to enhanced protein degradation in CKD patients[20]. Sulaiman SH 2021[18] reported significantly lower SOD activity in AKI patients compared with controls, which is contrary to our study; this may be due to increased antioxidative defense or to a small sample size in our study.

Ye Z et al.[21] found that expression of p53 was increased in patients with CKD, consistent with our study. Our study also indicated that p53 gene expression is significantly higher in CKD patients than in AKI patients (**Figure Ie)**. Additionally, p53 levels increase progressively with CKD stages, with the greatest upregulation observed in stages 4 and 5 compared with stages 2 and 3 (**Figure IIe)**. No contrary data were found. Zhen X et al.[22] reported that the accumulation of Nicotinamide N-methyltransferase (NNMT) in CKD may reduce p53 methylation while increasing p53 expression and activity.

Ming WH et al.[23] study showed that p53 regulates Wnt7a expression through transcription, activates the Wnt/β-catenin signalling pathway in UUO and adenine-induced renal fibrosis models, and participates in the process of renal fibrosis. Wnt9a significantly upregulated the

levels of ageing-related p16, p19, p53, and p21, promoting renal fibrosis in humans.[24] These studies show the association of p53 with the Wnt/β-catenin signaling pathway. Therefore, in CKD, p53 enhances Wnt/β-catenin signaling, which, in turn, increases p53 expression[23,24]. Lee C-C et al.[25] found that circulating eCyPA and uCyPA levels markedly increase in AKI. Similarly, Chatchawal et al.[26] found increased uCypA in patients with progression of renal deterioration, specifically in the diabetes with late-stage CKD group, compared with controls. These studies are consistent with our study.

In this study, the CyPA gene was upregulated in CKD and AKI patients compared with controls, with higher expression in AKI than in CKD (**Figure If)**. Chatchawal P et al.[26] reported elevated uCypA levels in patients with early-stage CKD. In contrast, in our study, the level of CypA levels in early-stage CKD was similar to that in controls (**Figure IIf).** The Chatchawal P et al.[26] study measured CypA protein (reduced clearance), whereas in our study, mRNA levels were measured. Despite growing evidence that oxidative stress and cellular defense mechanisms play a central role in CKD, there is a lack of local data and inconsistent global findings on the expression of of *KL*, *CAT, SOD1*, *p53,* and *CyPA* genes. Previous studies have reported variable expression trends, with some indicating upregulation and others showing marked downregulation during disease progression. These discrepancies, likely influenced by study design, disease stages, and methodological variations, limit our understanding of their true role in CKD pathogenesis. Ultimately, this study bridges the gap between molecular insights and clinical management by elucidating the expression patterns of genes involved in OS regulation and renal protection. Therefore, these findings highlight the involvement of these genes in the complex interplay of oxidative stress and cellular regulation, offering new insights into CKD pathogenesis and paving the way for innovative diagnostic and therapeutic strategies.

CONCLUSION

The relative expression of *KL*, *CAT, SOD1*, *p53* and *CyPA* genes reveals their crucial role in modulating OS during the pathogenesis of CKD. Their expression across advancing CKD stages suggests impaired antioxidant defense mechanisms, contributing to renal damage and disease progression. These findings underscore the potential of these genes as biomarkers for CKD severity and as therapeutic targets to mitigate OS and preserve kidney function.

Ethical permission: Institutional review board/ Independent ethical committee (IRB/IEC) No: C-29-970.
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Data Sharing Statement: The corresponding author can provide the data proving the findings of this study on request. Privacy or ethical restrictions bound us from sharing the data publicly.

AUTHOR CONTRIBUTION

Farooq B: Conceived, designed the study, collected data and finalized the manuscript.
Iqbal M: Assisted in patient recruitment and reviewed the manuscript for accuracy.
Jaffar M: Coordinated analysis and contributed to the literature review.
Qureshi ZH: helped with data management and formal analysis.
Faisal M: helped in conducting the experiments, data and statistical analyses, and manuscript writing.
Lodhi MS: helped in conception, conducting experiments and data analysis.

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