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Investigation of ferroptosis-related gene expression and oxidative stress markers in end-stage diabetic kidney disease: A case–control analysis

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ABSTRACT

End-stage renal disease (ESRD), the most advanced stage of chronic kidney disease, is a major diabetes-related complication associated with severe metabolic and oxidative disturbances. Ferroptosis, an iron-dependent form of regulated cell death, may contribute to diabetic kidney disease (DKD) progression; however, its role in ESRD remains unclear. This case-control study investigated oxidative stress markers and ferroptosis-related gene expression in ESRD patients secondary to DKD and healthy controls. Biochemical parameters, oxidative stress markers, and the mRNA expression of tumor suppressor genes *(p21* and *p53)* and nuclear factor erythroid 2-related factor 2 (*Nrf2)* were assessed in peripheral blood mononuclear cells. Group comparisons were performed using appropriate statistical tests, and logistic regression was used to evaluate the relationship between ESRD status. ESRD patients showed significantly higher *Nrf2* expression ($P < 0.0001$) and lower *p53* expression ($P = 0.026$), while *p21* expression did not differ significantly ($P = 0.32$). Catalase activity and malondialdehyde levels were significantly increased in ESRD ($P = 0.0001$), whereas glutathione peroxidase activity was decreased ($P = 0.018$). Logistic regression demonstrated significant associations between ESRD and CAT activity (OR = 1.082), MDA levels (OR = 3.627), GPx activity (OR = 0.985), *Nrf2* expression (OR = 2.133), and *p53* expression (OR = 0.711) (all $P < 0.05$). These findings suggest that ESRD secondary to DKD is associated with significant alterations in oxidative stress status and changes in the expression of genes linked to ferroptosis-related pathways, providing indirect evidence of their possible involvement.

Keywords: End-stage renal disease, Diabetic kidney disease, Ferroptosis, Gene expression, Oxidative stress

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Introduction

The prevalence of diabetes has more than doubled worldwide over the past 20 years, and individuals with diabetes have a 30–40% risk of developing diabetic kidney disease (DKD) (1). The pathogenesis and clinical manifestations of DKD are associated with marked structural alterations in the kidneys of patients with diabetes (1). DKD is still the most common cause of end-stage renal disease (ESRD). Hyperglycemia in diabetes induces oxidative stress in various tissues by generating reactive oxygen species (ROS), which play a significant role in ESRD pathogenesis. The kidney, a metabolically active organ, generates ATP primarily through mitochondrial oxidative phosphorylation driven by aerobic metabolism. While this process is essential for cellular function, it can produce ROS as by-products at various enzymatic complexes (2, 3).

Defects in this process result in inefficient electron transport, leading to ROS overproduction and oxidative stress. Impairment of mitochondrial respiration has been observed in patients with ESRD and in those receiving dialysis. It has been reported that under pathological conditions such as diabetes, excessive generation of ROS plays a central role in disrupting metabolic pathways in the kidneys, resulting in inflammation, fibrosis, endothelial dysfunction, and altered renal structure and function (4).

Regulating cell death is critical for proper development, the achievement of homeostasis, and understanding the mechanisms behind acute and chronic diseases. This process is often associated with inflammation, impaired cellular function, and tissue damage. Ferroptosis has been a recently recognized form of regulated cell death, a non-apoptotic form of cell death that differs from necroptosis, autophagy, and apoptosis. It involves iron-driven lipid peroxidation accompanied by excessive accumulation of ROS and is closely linked to dysregulated iron homeostasis and oxidative stress pathways (5). Ferroptosis has been increasingly implicated in the pathogenesis of several kidney diseases. Unlike other forms of cell death, ferroptotic cells display distinct genetic, biochemical, morphological, and metabolic characteristics. Ferroptosis can also propagate rapidly in a wave-like manner within cell populations. It is associated with specific mitochondrial abnormalities, including swelling, altered membrane density, and rupture of the outer mitochondrial membrane. The activation and progression of ferroptosis are governed by the convergence of three main regulatory axes, including iron homeostasis, glutathione levels, and lipid metabolic activity, especially within renal tubular epithelial cells (6).

Ferroptosis is a pathway closely linked to oxidative stress and various medical conditions, including acute renal injury, cancer, heart disease, neurological disorders, and liver disorders. In ESRD, increased ROS production and disrupted iron homeostasis have been proposed as mechanisms that may contribute to ferroptosis-related processes. Several factors associated with diabetes, including hyperglycemia, chronic inflammation, and oxidative stress, markedly increase ROS production, which can promote ferroptosis (7, 8).

Although factor erythroid 2-related factor 2 (*Nrf2)* was originally believed to have an anti-ferroptotic action mainly via controlling the antioxidant response, studies have shown that *Nrf2* also has anti-ferroptotic effects through controlling important facets of lipid and iron metabolism (9). Both wild-type and tumor-associated mutant tumor protein p53 *(p53)* can regulate ferroptosis, and the first study examining *p53*'s role in ferroptosis and tumor suppression revealed that SLC7A11 is a direct target gene inhibited by *p53* (10). The *p53* transcriptional target cyclin-dependent kinase inhibitor 1A *(p21)* inhibits ferroptosis by maintaining reduced glutathione (GSH) levels (11). Eryptosis, the programmed death of red blood cells (RBCs), shares certain features with ferroptosis, including oxidative injury, calcium influx, and exposure of phosphatidylserine on the membrane, and involves pathways such as ceramide formation and caspase activation (12).

Several studies have reported alterations in oxidative stress biomarkers, including malondialdehyde (MDA), catalase (CAT), and glutathione peroxidase (GPx), in patients with kidney disease (13, 14). In addition, emerging evidence suggests that ferroptosis, a form of iron-dependent regulated cell death, contributes to the development and progression of kidney injury (15, 16). Despite these findings, studies investigating oxidative stress and ferroptosis simultaneously in human subjects remain limited. Most previous research has focused either on oxidative stress biomarkers or on ferroptosis-related mechanisms, particularly in experimental models. Moreover, the association between ferroptosis-related

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genes, such as *Nrf2*, *p53*, and *p21*, and oxidative stress status in patients with end-stage DKD has not been fully clarified. Therefore, the present study aimed to evaluate oxidative stress biomarkers and the expression of ferroptosis-related genes (*p53*, *p21*, and *Nrf2*) in patients with ESRD and to compare the findings with those of healthy controls.

Methods

Participants

This case-control study was designed to compare ferroptosis-related gene expression and oxidative stress markers between patients with ESRD secondary to DKD and healthy control subjects without a history of diabetes or kidney disease. A total of 100 individuals were enrolled, including 50 patients with ESRD attributable to DKD who were receiving maintenance hemodialysis at the Hemodialysis Center of Tabriz University of Medical Sciences and 50 healthy controls. The diagnosis of ESRD was made based on clinical history, laboratory findings, and nephrologist evaluation.

All of the patients were on regular hemodialysis three times a week, and each session lasted about four hours. Blood samples were taken immediately before the dialysis session to avoid the acute effects of dialysis. The control group included healthy volunteers with no history of diabetes, kidney disease, cardiovascular disorders, autoimmune diseases, infections, inflammatory conditions, or malignancy. Patients with documented alternative causes of ESRD were excluded. Controls were selected to be broadly similar to the patient group in terms of age and sex. All participants were clinically evaluated before enrollment. Other factors such as body mass index, smoking, medications, diet, and physical activity were not matched between the two groups, which should be considered when interpreting the results. Exclusion criteria included transplanted individuals, pregnant women, those with liver disease or a history of malignancy, and individuals with significant comorbidities.

Sampling and laboratory measurements

5.0 mL of peripheral venous blood samples were collected from all participants using both EDTA-containing tubes and serum-separator gel tubes. Peripheral blood mononuclear cells (PBMCs) were isolated from EDTA-anticoagulated blood samples using density-gradient centrifugation and were subsequently used for total RNA extraction and gene expression analyses. Erythrocytes were also obtained from EDTA blood samples and were used for the assessment of eryptosis by flow cytometry. Serum was separated from blood samples collected in serum separator tubes by centrifugation and subsequently used for the determination of oxidative stress biomarkers. CAT activity was measured using the KPG-CAT Activity Assay Kit (Biotechnology Research Company, Iran), MDA levels were determined using the KPG Lipid Peroxidation (MDA) Assay Kit (Biotechnology Research Company, Iran), and GPx activity was assessed using the GPx Activity Assay Kit (Navand Salamat, Iran), all according to the manufacturers' instructions. The CAT and GPx assays measured enzymatic activity rather than protein concentration. Routine biochemical parameters, including fasting blood sugar (FBS), creatinine, triglycerides, total cholesterol, calcium, urea, uric acid, and iron (Fe), were measured using standard clinical laboratory methods routinely employed in the hospital laboratory.

RNA isolation

Total RNA was isolated from PBMCs using TRIzol reagent (Invitrogen, Carlsbad, CA). Briefly, isolated PBMCs were lysed in TRIzol reagent according to the manufacturer's instructions. Chloroform was added for phase separation, followed by isopropanol precipitation of RNA. The separated RNA was washed twice with 70% ethanol, dried at 25 °C, and dissolved in 15 µL of DEPC–treated water. RNA concentration and quality were checked with a Nanodrop spectrophotometer (A_{260}/A_{280} ratio ~1.8–2.0) and confirmed by 2% agarose gel electrophoresis.

cDNA synthesis

The RevertAid First Strand TaKaRa cDNA Synthesis Kit (Kusatsu, Japan) was used for complementary DNA (cDNA) synthesis on an Eppendorf Mastercycler Gradient (Hamburg, Germany). A 15 µL reverse transcription reaction contained 1.0 µg of total RNA, 0.5 µL of Oligo(dT) primer, 0.5 µL of random hexamer primer, 0.5 µL of dNTP mix, 4.0 µL of 5X RT buffer, 1.0 µL of reverse transcriptase (RT) enzyme, and DEPC-treated water to a final volume. RNA-primer mixtures were denatured

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at 65 °C for 5 minutes and immediately chilled on ice for 2 minutes. After a brief centrifugation, the prepared solution was incubated at 65 °C for five minutes. The random hexamer and oligo(dT) primers were then incubated for 60 minutes at 37 °C and 42 °C, respectively. Before starting the reaction, the mixture was incubated in a thermocycler at 70 °C for five minutes. As a negative control, a reaction containing all cDNA synthesis components except RNA was included to monitor contamination during the synthesis process.

Quantitative real-time PCR (qRT-PCR)

The expression of *Nrf2*, *p53*, and *p21*, along with the housekeeping gene β2-microglobulin (*B2M*; used as an endogenous reference), was quantified using SYBR Green–based quantitative real-time PCR (qRT-PCR). B2M was used as the endogenous reference gene because it has been widely employed as a normalization control in gene expression studies using peripheral blood mononuclear cells (PBMCs) and showed minimal variation in Ct values across the analyzed samples. The reactions (final volume of 20 µL) were performed on a commercial PCR (polymerase chain reaction) device (Roche Molecular Systems, Inc., Pleasanton, CA, USA). The sequences of the primers used are presented in Table 1. The qRT-PCR conditions included an initial denaturation at 94 °C for 10 minutes (one cycle), followed by 40 cycles of 95 °C for 15 seconds (denaturation), 60 °C for 40 seconds (annealing), and 72 °C for 40 seconds (extension), with a final extension at 72 °C for 5 minutes.

Table 1. Primer sequences used in the present work.

Gene	Primer sequence
p53	Forward: 5′-ATCTACAAGCAGTCACAGCAC-3′
	Reverse: 5′-TACAGTCAGAGCCAACCTCAG-3′
Nrf2[a]	Forward: 5′-GGCATCACCAGAACACTCAGT-3′
	Reverse: 5′-GTGCTCTGCCCCTGAGATG-3′
p21	Forward: 5′-CCCGTGAGCGATGGAACTT-3′
	Reverse: 5′-CTGCCTCCTCCCAACTCATC-3′
B2M[a]	Forward: 5′-GAGTATGCCTGCCGTGTGAA-3′
	Reverse: 5′-TGCGGCATCTTCAAACCTCC-3′

[a]*Nrf2* (Nuclear factor erythroid 2-related factor 2) and *B2M* (β 2 microglobulin).

Flow-cytometric assay of cell death in RBCs using FITC Annexin V

The flow-cytometric assay based on FITC-Annexin V was used to evaluate eryptosis in blood cells of ESRD patients using the FITC Annexin V apoptosis detection kit (ebioscience, USA). For this purpose, peripheral blood samples were obtained from all participants and centrifuged. Subsequently, the separated RBCs were washed with phosphate-buffered saline (PBS, pH 7.4). The cells were then incubated with 1.0 µL of Annexin V-FITC. Finally, Annexin V fluorescence intensity was recorded by excitation of the samples at 488 nm using a flow cytometer (FACS Calibur flow cytometer; BD Biosciences, San Jose, CA).

Statistical evaluation

The data analysis was performed using SPSS Version 25 software. All variables were tested for normality using the Shapiro-Wilk test. Comparisons between ESRD and the control group were carried out using the t-test or Mann-Whitney U test based on data

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normality. Relative gene expression was calculated using the $2^{-\Delta Ct}$ method, where ΔCt was obtained by subtracting the Ct value of the reference gene (*B2M*) from the Ct value of the target gene. Gene expression variables were log-transformed before logistic regression analysis to reduce skewness and improve model stability. Effect sizes were calculated using standardized mean differences (SMDs) to quantify the magnitude of differences between the two groups. Binary logistic regression analysis was performed to assess the association of oxidative stress markers and ferroptosis-related gene expression with ESRD status, where ESRD status was defined as the dependent variable (ESRD = 1, control = 0). Results were reported as odds ratios (ORs) with 95% confidence intervals (95% CIs) and corresponding P-values. In addition, Spearman's rank correlation analysis was used to evaluate the relationships between oxidative stress markers and ferroptosis-related gene expression within the ESRD group. Correlation coefficients (r) and P-values were calculated, and statistical significance was set at $P < 0.05$. Receiver operating characteristic (ROC) curves were used to evaluate the potential of the genes as biomarkers. The results were reported as mean ± standard deviation (SD) for each parameter.

Results

Biochemical parameters analysis

Clinical data and various biochemical parameters were compared between ESRD patients and control subjects. According to Table 2, there were no significant differences in age distribution between the two groups.

Table 2. The clinical and laboratory features of participants and control subjects.

Parameters	Control	ESRD	*P*-value [b]
Blood pressure	122/70	-	-
HbA1c (%)	5.50 ± 1.31	7.35 ± 1.67	0.001***
Age (years)	49.98 ± 14.37	51.93 ± 13.66	0.428
[a]FBS (mg/dL)	85.18 ± 6.53	126.43 ± 18.45	0.001**
Creatinine (mg/dL)	1.17 ± 0.91	8.74 ± 2.63	0.001**
Cholesterol (mg/dL)	151.70 ± 27.96	153.81 ± 25.65	0.781
Triglycerides (mg/dL)	126.90 ± 25.75	182.52 ± 38.49	0.001**
Urea (mg/dL)	30.29 ± 7.14	108.02 ± 14.39	0.001**
Uric acid (mg/dL)	4.77 ± 0.98	5.23 ± 1.35	0.054
Fe (µg/dL)	86.31 ± 14.42	99.57 ± 13.48	0.001**
Calcium (mg/dL)	9.11 ± 0.59	8.97 ± 0.77	0.566
CAT[a] (U/L)	227.08 ± 29.95	339.05 ± 31.33	0.001**
MDA[a] (nmol/mL)	2.78 ± 0.80	6.84 ± 1.69	0.001**
GPx[a] (U/L)	242.56 ± 28.72	231.88 ± 28.99	0.018*

FBS (Fasting blood sugar), CAT (catalase), MDA (Malondialdehyde), and GPx (Glutathione peroxidase). [b] Data are presented as mean ± SD. The Shapiro-Wilk test was utilized to assess normality. For comparisons of continuous variables, either Student's t-test or appropriate non-parametric methods were employed. $P < 0.05$ was regarded as statistically significant. $P < 0.05$ (*), and $P < 0.001$ (**) indicate levels of statistical significance.

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The mean ages of ESRD patients and the control group were comparable, at 51.93 ± 13.66 years (range: 30–85) and 49.98 ± 14.37 years (range: 20–80), respectively. The ESRD group showed markedly elevated serum creatinine (8.74 ± 2.63 mg/dL vs. 1.17 ± 0.91 mg/dL) and urea levels (108.02 ± 14.39 mg/dL vs. 30.29 ± 7.14 mg/dL), consistent with ESRD.

Expression of *p53*, *Nrf2*, and *p21* in patients and controls

Statistical analysis showed a substantial downregulation of *p53* expression in the ESRD patients when compared to the reference group (P = 0.026). *Nrf2* expression was considerably upregulated in patients relative to controls (P = 0.0001). However, no significant difference was observed in *p21* expression between the studied groups (P = 0.32; Figure 1).

The diagnostic potential of *p53*, *Nrf2*, and *p21* was evaluated in patients and controls using ROC curve analysis (Figure 2). The ROC analysis revealed that *p53* and *Nrf2* had area under the curve (AUC) values of 0.63 (P = 0.143) and 0.75 (P < 0.0001), respectively. For *p53*, the sensitivity and specificity were 67% and 58%, while *Nrf2* showed a sensitivity of 67% and a specificity of 73%. These results indicate that both markers have limited diagnostic performance and may serve as poor standalone biomarkers in this patient population.

Associations of oxidative stress markers and gene expression with ESRD status

Comparisons between ESRD patients and healthy individuals revealed varying degrees of differences in oxidative stress markers and gene expression. Among the investigated genes, *Nrf2* expression showed a small-to-moderate difference between groups (SMD = 0.38), whereas *p21* expression demonstrated only a slight difference (SMD = 0.325). In contrast, *p53* expression showed a moderate difference between ESRD patients and controls (SMD = 0.526). Oxidative stress markers showed much more pronounced differences between groups. CAT activity was significantly higher in ESRD patients (SMD = 3.7), and serum MDA levels were also markedly elevated (SMD = 3.087). GPx activity showed a moderate difference between groups (SMD = 0.435).

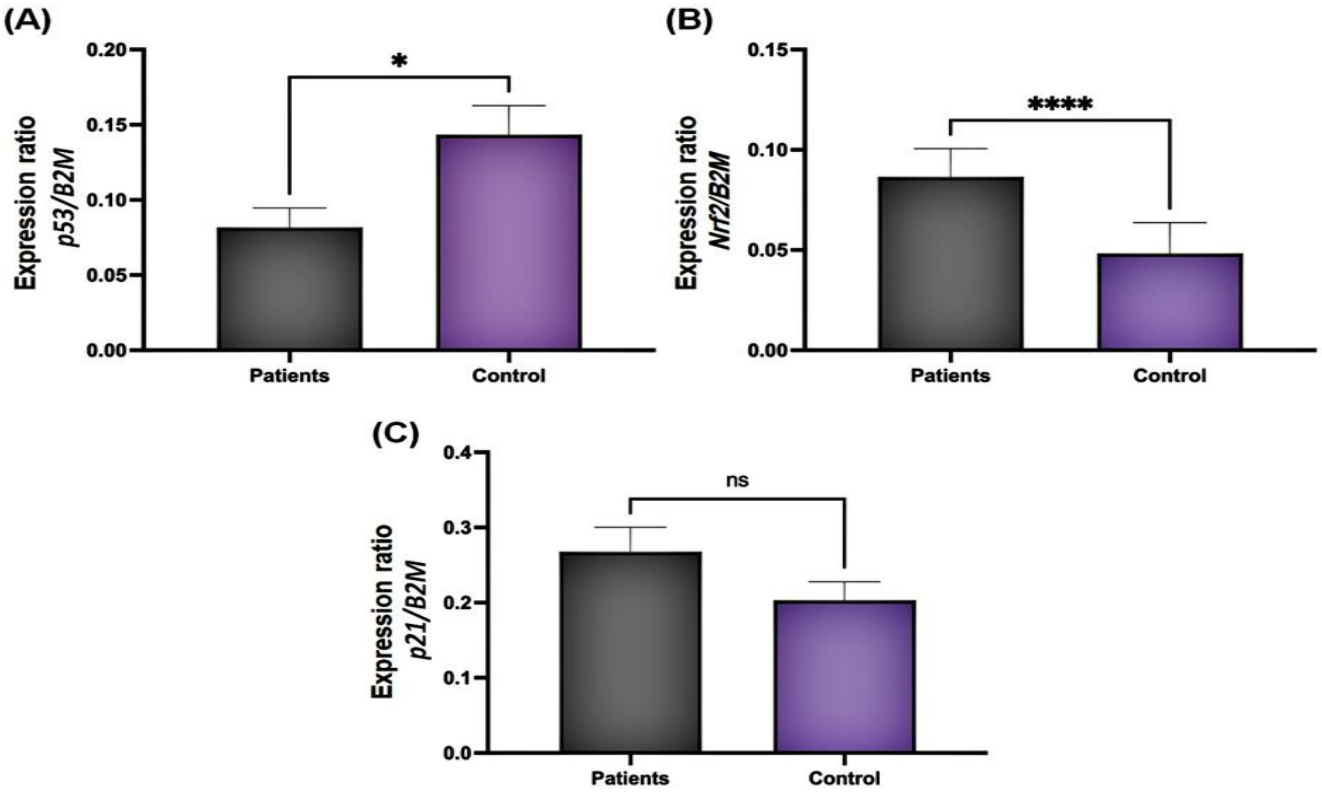

Figure 1. Relative expression of *p53* (A), *Nrf2* (B), and *p21* (C) in ESRD patients compared with healthy controls.

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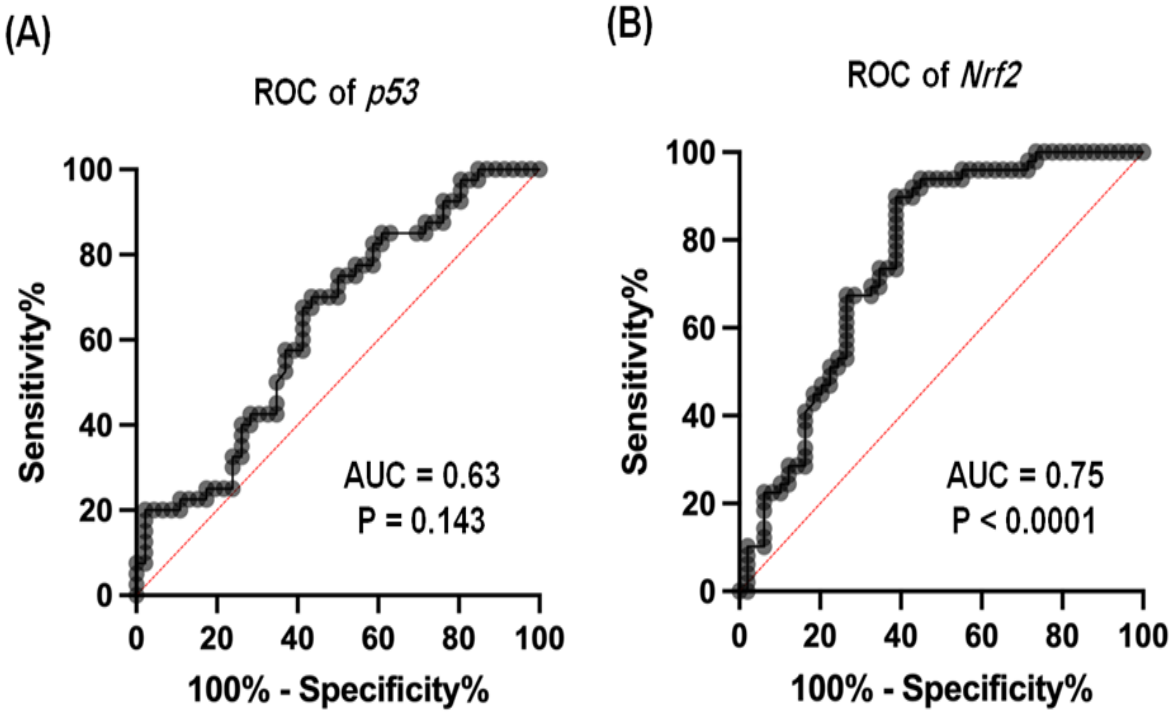

Figure 2. ROC curve analysis of *p53* and *Nrf2* for assessing their biomarker potential in patients.

While SMDs describe the magnitude of group differences, logistic regression analysis was subsequently performed to evaluate the association of each variable with ESRD status. In univariate logistic regression analysis,

CAT activity (OR = 1.082, 95% CI: 1.041–1.124, P < 0.0001), serum MDA levels (OR = 3.627, 95% CI: 2.074–6.344, P < 0.0001), *Nrf2* gene expression (OR = 2.133, 95% CI: 1.454–3.129, P < 0.0001), and *p53* gene expression (OR = 0.711, 95% CI: 0.507–0.996, P = 0.047) were significantly associated with ESRD status. No significant association was observed for *p21* expression.

GPx activity showed a moderate difference between groups and remained significantly associated with ESRD status in logistic regression analysis, although the magnitude of the association was relatively small (OR = 0.985, 95% CI: 0.970–1.000, P = 0.049). In addition, correlation analysis was performed within the ESRD group to investigate the relationship between oxidative stress markers and ferroptosis-related gene expression. As presented in Table 3, significant positive associations between Nrf2 expression and several oxidative stress markers, including MDA (r = 0.250, P = 0.012), GPx (r = 0.322, P = 0.0011), and CAT (r = 0.377, P = 0.0001). No significant correlations were observed between Nrf2 expression and serum iron levels. In contrast, p53 and p21 expressions were not significantly correlated with any of the investigated oxidative stress markers (Table 3).

Eryptosis study

The externalization of phosphatidylserine (PS) on the outer membrane of RBCs serves as a signal for suicidal erythrocyte death, or eryptosis, which is important for clearance from the bloodstream. RBC eryptosis in ESRD patients was assessed using Annexin V staining by flow cytometry, and the findings are presented in Figure 3.

As shown, the percentage of Annexin V-positive cells (representing early apoptosis or eryptosis) was markedly higher in patients (18.2%) when compared with the control group (0.15%), confirming that eryptosis is enhanced in ESRD patients, potentially due to oxidative stress

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Table 3. Correlation analysis between oxidative stress markers and ferroptosis-related gene expression in patients with ESRD.

Variable	*Nrf2*	*p53*	*p21*
MDA	r = 0.250, P = 0.012	r = -0.147, P = 0.143	r = 0.011, P = 0.907
GPx	r = 0.322, P = 0.0011	r = -0.077, P = 0.442	r = 0.062, P = 0.533
CAT	r = 0.377, P = 0.0001	r = 0.006, P = 0.949	r = 0.150, P = 0.135
Fe	r = 0.180, P = 0.0719	r = 0.069, P = 0.491	r = 0.078, P = 0.436

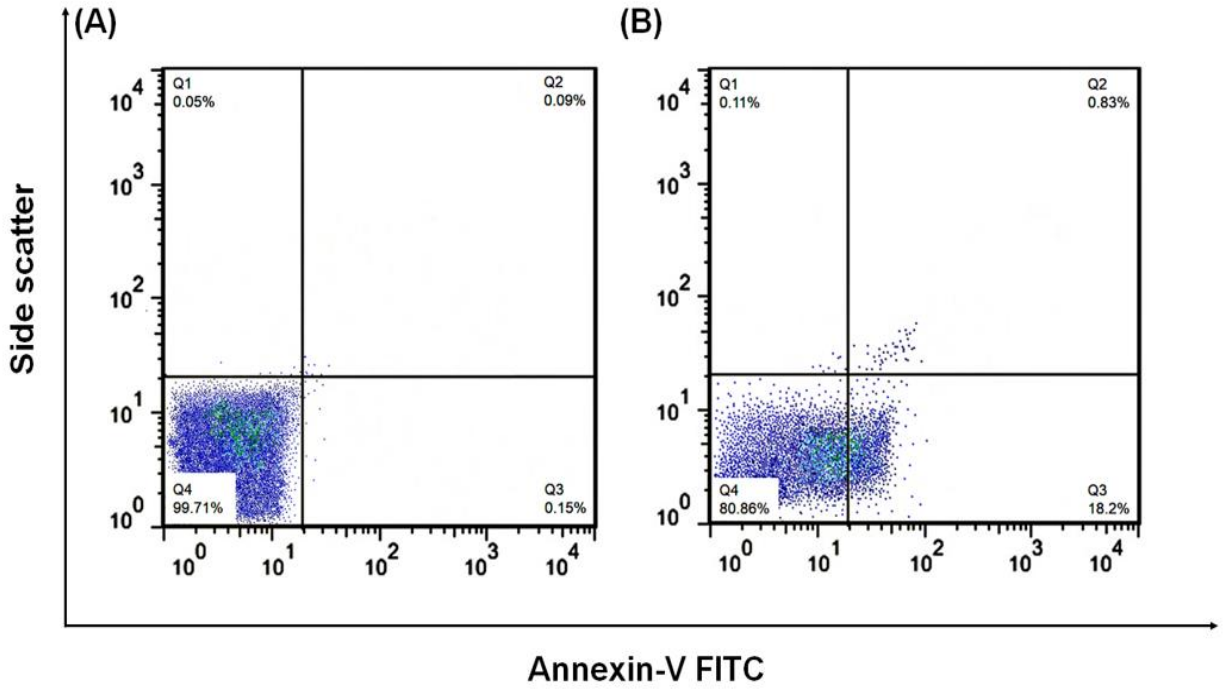

Figure 3. Flow cytometric analysis of eryptosis in RBCs. (A) Control group and (B) RBCs from ESRD patients.

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Discussion

An important finding of the present study was the significant correlation observed between oxidative stress markers and ferroptosis-related gene expression in patients with ESRD. These results suggest that elevated oxidative stress may be linked to alterations in ferroptosis-related regulatory pathways. Given the well-established role of oxidative stress in promoting lipid peroxidation and iron-dependent cell death, our findings support the potential involvement of ferroptosis-related mechanisms in ESRD. Previous studies have reported the involvement of ferroptosis in kidney diseases, emphasizing its function in various physiological and pathological processes (7). The novelty of the present study lies in the combined assessment of classical oxidative stress markers together with ferroptosis-related gene expression in patients with end-stage DKD. While previous investigations have largely focused on oxidative stress parameters or ferroptosis mechanisms separately, our study evaluated both pathways simultaneously in a clinical setting. Our findings indicated that serum levels of Fe and MDA were significantly elevated in patients with ESRD. Excess Fe in the blood can undergo the Fenton reaction with ROS to produce •OH radicals that promote lipid peroxidation (7). These processes have been implicated in ferroptosis-related pathways; however, serum iron alone cannot be considered a specific marker of ferroptosis. Furthermore, enzymatic antioxidants, including GPx, CAT, and superoxide dismutase (SOD), are crucial in regulating iron-dependent lipid peroxidation (17). Among these, the system Xc–GSH–GPx4 pathway serves as the primary defense mechanism against ferroptosis. System Xc is a cell membrane cystine-glutamate antiporter that facilitates glutathione (GSH) synthesis by combining cysteine with glutamic acid and glycine (18). Inhibition of system Xc activity is a primary trigger of ferroptosis, as it reduces intracellular cysteine levels, leading to GSH depletion, reduced glutathione peroxidase 4 (GPx4) performance, and subsequent lipid peroxidation. GPx includes multiple isoenzymes, with GPx4 being a distinct and important regulator of ferroptotic cell death (19). Our data indicated that in ESRD patients, GPx catalytic activity was significantly decreased, whereas Fe levels were significantly increased. Additionally, serum MDA concentrations were markedly elevated. Although these findings are consistent with biochemical changes reported in ferroptosis-related pathways, the present study did not evaluate established ferroptosis-specific markers such as GPx4, SLC7A11, ACSL4, ferritin, transferrin receptor, glutathione, or lipid ROS. Therefore, the observed findings should be interpreted as indirect evidence of the possible involvement of ferroptosis-related pathways rather than confirmation of ferroptotic cell death. Accordingly, the present study does not directly demonstrate ferroptosis but rather identifies alterations in oxidative stress markers and ferroptosis-related regulatory genes that may be relevant to ferroptosis-associated mechanisms in ESRD.

GPx4 is considered a critical regulator of ferroptosis. It should be noted that the present study measured total GPx enzymatic activity rather than GPx4 specifically; therefore, no direct conclusions regarding GPx4 status can be drawn. Reduced GPx4 activity has been reported to impair the detoxification of lipid peroxides and has been associated with increased susceptibility to ferroptosis in experimental models (20, 21).

Different studies have shown that knockout of GPx4 in mice leads to the enhancement of lipid oxidation in all examined models, showing the importance of this enzyme in protecting cells from lipid peroxide–induced damage (21, 22). Yang et al. (23) discovered that ferroptosis is inhibited by upregulation of GPx4 expression, whereas cells with downregulated GPx4 are more susceptible to ferroptosis. Furthermore, RSL3 decreases cellular antioxidant capacity and increases ROS accumulation by directly targeting and inhibiting GPx4, ultimately inducing ferroptosis (24). These findings are consistent with our observations of altered oxidative stress markers and gene expression patterns that may be related to ferroptosis-associated pathways in ESRD patients.

Despite the decrease in GPx activity, we observed an increase in CAT activity in patients compared to the control group. Elevated CAT activity may reflect a compensatory response to increased hydrogen peroxide levels in ESRD patients. This increase may be an attempt by the body's defense system to decrease the harmful impacts of oxidative stress, but if the production of oxidative species exceeds the capacity of the defense systems, even the increase in CAT activity will not be enough, and oxidative damage will occur. Overall, these findings suggest alterations in the

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antioxidant defense system of ESRD patients, which may contribute to disease progression. Strategies aimed at regulating Fe levels and enhancing the activity of antioxidant enzymes could be beneficial in managing this condition (25, 26).

Additionally, Annexin V fluorescence was increased in the RBCs of ESRD patients, indicating higher rates of eryptosis. Elevated Annexin V fluorescence indicates increased PS externalization and is consistent with higher levels of eryptosis in ESRD patients. This observation may be related to the altered oxidative stress profile observed in this group.

In addition, we investigated the expression of key genes involved in ferroptosis, including *p21*, *p53*, and *Nrf2*. We observed significantly higher *Nrf2* expression and lower *p53* expression in ESRD patients, whereas the increase in *p21* expression did not reach statistical significance. *p53* is a central regulator of cellular stress responses and apoptosis, and it also plays a critical role in ferroptosis regulation (27). Under normal conditions, *p53* functions as a tumor suppressor and helps maintain cellular stability by regulating genes involved in oxidative damage and lipid peroxidation (28). *p53* regulates ferroptosis in a complex, context-dependent, and tissue-specific manner, with the ability to both promote and inhibit ferroptosis. These pathways are classified as canonical or noncanonical depending on whether they rely on GPx4 (10, 29). The protein *p53* stops ferroptosis, a specific type of cell death, by directly blocking the enzyme DPP4. The involvement of *p53* in controlling ferroptosis has been demonstrated across many studies to be context-specific (29). However, the observed reduction in *p53* expression in our patients may be consistent with alterations in ferroptosis-related regulatory pathways.

Nrf2 acts as a key transcription factor in modulating the antioxidant response of cells and inhibits ferroptosis by regulating various molecules, such as glutamate cysteine ligase (GCL), glutathione synthase (GSS), and GPx4. Therefore, *Nrf2* has been suggested as an important regulator of ferroptosis, with ferroptosis-related proteins being directly or indirectly modulated by its activity (30). *Nrf2* regulates GPx4 synthesis and function, as well as intracellular Fe levels, through the *Nrf2*-HO-1 axis. This pathway can have dual effects under oxidative stress and Fe accumulation, potentially either the induction or inhibition of ferroptosis depending on the duration and intensity of Fe exposure (30). The *Nrf2*-HO-1 axis protects cells against ferroptosis during short-term Fe accumulation, but prolonged Fe exposure may result in increased Fe accumulation and heightened susceptibility to ferroptosis (31). Patients with ESRD have been reported to show higher levels of oxidative stress and alterations in Fe homeostasis compared with healthy controls. These alterations may be associated with disease status and have been proposed to contribute to increased ROS production and ferroptosis-related pathways. Kidney dysfunction has been associated with iron imbalance and increased levels of free iron, which may be linked to ROS accumulation, oxidative stress, and ferroptosis-related processes.

A limitation of this study is that all participants were patients with ESRD undergoing maintenance hemodialysis. ESRD involves complex metabolic, inflammatory, and oxidative changes that may independently affect both gene expression and oxidative stress markers. Therefore, the observed alterations may reflect the combined effects of disease progression and dialysis rather than ESRD alone. Future studies including patients at different stages of kidney disease would help clarify the role of ferroptosis-related pathways more precisely. Another limitation is that only age and sex were matched between groups. Other factors such as BMI, smoking, medication use, diet, and physical activity were not fully controlled, which may have influenced the results. Gene expression analyses were performed using PBMC-derived RNA, which provides a readily accessible source of systemic molecular information and has been widely used to investigate oxidative stress and ferroptosis-related pathways in human diseases. However, PBMC gene expression may not fully reflect molecular changes occurring within renal tissue. Finally, ferroptosis was not directly measured in this study. Although we assessed oxidative stress markers and genes related to ferroptosis, we did not evaluate specific markers of ferroptotic cell death. For this reason, our findings should be interpreted as indirect evidence rather than confirmation of ferroptosis involvement in ESRD. Further experimental studies are needed to address this issue.

The results of this study demonstrated that ESRD patients experience severe oxidative stress, resulting in significant alterations in oxidative stress markers and in genes that have been implicated in ferroptosis-

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related pathways. Elevated Fe and MDA levels, along with decreased GPx activity and increased CAT activity, indicate an imbalance in the antioxidant system that may contribute to disease progression. Moreover, the increased expression of Nrf2, a master regulator of cellular antioxidant defense, likely represents an adaptive response to heightened oxidative stress. In contrast, the reduced expression of p53 may indicate dysregulation of cellular stress-response pathways, which are increasingly recognized as important modulators of ferroptosis. Moreover, the increased expression of *Nrf2*, a key regulator of cellular antioxidant defense, likely reflects an adaptive response to heightened oxidative stress. In contrast, the reduced expression of *p53* may indicate dysregulation of cellular stress-response pathways, which are increasingly recognized as important modulators of ferroptosis. These findings support a possible involvement of ferroptosis-related pathways in ESRD and should be considered hypothesis-generating pending confirmation in mechanistic studies.

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