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Article

Ferroptosis-Related Lipid Peroxidation Biomarkers as Early Predictors of Diabetic Kidney Dysfunction

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Abstract: Diabetic kidney disease (DKD) is a major cause of end-stage renal disease (ESRD) worldwide. This condition is significantly more prevalent among Iraqis. The potential of ferroptosis as a biomarker for early prediction of kidney dysfunction has not yet been explored in a Middle Eastern cohort. Ferroptosis is an iron-dependent form of regulated cell death driven by lipidd peroxidation. Further, an increasing number of literature report ferroptosis to be a pathogenic mechanism in DKD. To achieve this, 120 patients with T2DM who had never had kidney disease were included in the study. Blood specimens were obtained from them to determine kidney function and the concentrations of ferroptosis-related biomarkers. Moreover, we correlated these biomarkers with the severity of kidney disease in T2DM patients. Among total 165 participants, we included 40 healthy controls (HC), 45 T2DM without DKD, 42 early DKD, and 38 advanced DKD. Quantification of serum and urinary biomarkers was done using ELISA and colorimetric assessment methods. The kidney functions were assessed using estimated GFR, UACR, serum creatinine and blood urea nitrogen. ROC analysis assessed the accuracy of the prediction. Increase in MDA, 4-HNE, ACSL4 and TFR1 across various stages of the disease; Decrease in GPX4 and GSH (all $p<0.001$) This combined biomarker panel (MDA, 4-HNE, GPX4, and ACSL4) achieved an AUC of 0.928 (95% CI: 0.886–0.970), which was better than any of the individual biologic markers just taken alone. MDA is the most negatively correlated with eGFR and positively correlated with UACR. To sum up, it is evident that ferroptosis-associated lipid peroxidation biomarkers, particularly a combined panel, are highly predictive of early diabetic kidney dysfunction in the Iraqi population, and hence can be clinically actionable early detection tools.

Keywords: Ferroptosis, Lipid Peroxidation, Diabetic Kidney Disease, Biomarkers, Early Prediction, Iraqi Population, GPX4, ACSL4.

Introduction

Diabetic renal impairment (DKD) was at one time alluded to as diabetic nephropathy. This diabetic confusion is the main microvascular intricacy of diabetes mellitus. DKD is the essential reason for end-stage renal illness or ESRD around the world [1]. In 2021, an estimated 537 million adults had diabetes, and by 2045, this number is estimated to increase to 783 million [2]. The biggest rise in relative terms, however, is expected in the Middle East and North Africa region [2]. In Iraq, this problem is

particularly alarming. A recent cross-sectional study conducted in Basra reported a chronic kidney disease prevalence of 49.9% among T2DM patients. The global average is 28% [3]. A lack of cancer screening and local healthcare due to the conflicts in the Mediterranean basin over the last 30 years is the main cause of this proliferation.

The onset of Diabetic Kidney Disease was until now considered to be originated from oxidative stress resulting from high blood sugar levels, deposition of advanced glycation end products and functional irregularities of glomerulus inside the kidney. The built model could not clarify the complete field wound obtained in diabetic patients, particularly renovascular fibrosis improvement, and tubular cell demise started before the infection occurs, which frames the base for elective pathways of fibrosis. The discovery of ferroptosis, an iron-dependant regulated cell death related to lipid peroxidation, described by Dixon et al. [4], brought a fundamental change in understanding cell death mechanisms in DKD. Ferroptosis involves mitochondrial shrinkage (because of this, it is distinct from apoptosis, necroptosis, or pyroptosis), increased membrane density, and accumulation of lethal lipid hydroperoxides in cellular membrane (The lehninger principle 5th edition).

The Introduction of Ferroptotic Cascade is arisen when there is a disruption in the balance between lipid peroxidation and the antioxidant defense. Iron overload upregulates transferrin receptor 1 (TFR1) in the kidney, resulting in a Fenton reaction and hydroxyl radical generation. The generated radical, in turn, attacks polyunsaturated fatty acids (PUFAs) present in phospholipids of the membrane. Arachidonic acid and adrenic acid is esterified in preference to long-chain fatty acids by the acyl-CoA synthetase long-chain family member 4 (ACSL4) enzyme. Meanwhile, lipid hydroperoxides are decreased by the enhancer glutathione peroxidase 4 (GPX4) that supplements the kidney cells to detoxify. The final items from the oxidation of lipids is malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE). They function as final items of cell death. At the same, these products can be measured in blood and urine from body fluids. [5]

The researchers working on the different aspects of the DKD disease so far were able to figure out the impact of ferroptosis on the development of damaging kidney diseases. In an observational study conducted by Zhang et al. [6] in the future tense describes that the combined panel of GPX4, ACSL4, MDA, and ROS could be used to predict DKD in Chinese people but has not been able to be researched in a population of Middle Eastern people where the Prevalence and pattern of progression of DKD varies tremendously. Iraqi is a very suitable inhabitant to search for such elements DOS to the high percentage of T2DM unambiguous, the increased DTE of CDK, rick indigenous food containing oxidised lipids by conventional techniques and, finally, a high percentage of iron an anemias with aubination as well as unmonitored replacement treatment, all these factors further increase kidney IBs and also provide enough soft fiberotic pathway detour kidney XX.

Thus, this investigation was configured to compute the serum and urinary levels of ferroptosis related lipid peroxidation biomarkers (MDA, 4-HNE, GPX4, ACSL4, TFR1, and GSH) across all the stages diabetic kidney diseases in Iraqi population, the link between the biomarkers and the standard formulas of kidney function. Lastly, the accuracy of the single or combined panels to detect the condition at an early stage. In our work, we have forecasted that biomarkers of lipid peroxidation be related to kidney damage before it becomes apparent from the signs of eGFR. In addition to this, there will be a significant decline in the eGFR at the onset of the symptoms of DKD. It will be a smart victory, too.

2. Literature Review

2.1 Ferroptosis: Molecular Mechanisms and Renal Implications

Ferroptosis is a unique form of regulated cell death that is different (genetically, biochemically, and morphologically) from apoptosis, necrosis, and autophagy. This was discovered by Dixon et al. [4]. Key characteristics are range of iron dependence, lipid hydroperoxide accumulation and incapacity of the cell to detoxify these harmful species by means of the glutathione-GPX4 axis [7]. Importantly, the canonical ferroptotic pathway is activated upon the inhibition of the system Xc^- (SLC7A11/SLC3A2 heterodimer), a cystine/glutamate antiporter, thus causing intracellular cysteine depletion and reduction of glutathione (GSH) biosynthesis. GSH is required as a cofactor by GPX4 to reduce lipid

hydroperoxides to their alcohols and GSH depletion shuts down the main enzyme-based defence against lipid peroxidation [8].

The kidney is especially prone to ferroptosis due to several structural and functional features. Cells in the proximal tubule are the major site for reabsorptive function. They contain abundant mitochondria for ATP-dependent transport. They can be easily damaged by mitochondrial lipid peroxidation [9]. Moreover, the kidney is integral to recycling iron via the breakdown of transferrin and heme proteins that are filtered, which means iron levels are always high in the kidney [10]. The diabetic kidney suffers from all these vulnerabilities, i.e., hyperglycemia increases intracellular iron accumulation by increasing TFR1 and decreasing ferritin while also generating large amounts of reactive oxygen species owing to mitochondrial dysfunction and activation of NADPH oxidase [11], [12].

2.2 Lipid Peroxidation Biomarkers in Diabetic Kidney Disease

Decomposition of lipids creates a variety of reactive aldehydes with both mediating cellular damage and measurable biomarkers. The most assayed lipid peroxidation product, MDA is formed by decomposition of arachidonic acid and larger PUFAs and is generally assessed through the thiobarbituric acid reactive substances (TBARS) assay or, more in detail, by high-performance liquid chromatography (HPLC) [5]. 4-HNE is a α,β- unsaturated aldehyde which results from peroxidation of omega-6 PUFA. This compound, more biologically relevant than MDA, forms adducts with proteins disrupting cellular signaling and inhibits GPX4 activity which leads to further ferroptotic cell death [13], [14]. According to clinical studies, MDA and 4-HNE levels are greater in patients with DKD compared to diabetic patients without nephropathy and healthy controls. Furthermore, they progress as the disease progresses [15], [16].

The GPX4 and ACSL4 enzymes that regulate ferroptosis are informative biomarkers. The enzyme GPX4 is the only enzyme that can reduce complex lipid hydroperoxides in biological membranes. It is downregulated in renal tubular cells in diabetics, while deletion of its gene (in mice, for example) reproduces the ferroptotic phenotype [8], [17]. On the other hand, in DKD, upregulated ACSL4 correlates with the severity of disease and enriches cell membranes with oxidation-susceptible PUFAs. The transferrin receptor TFR1 (cellular iron uptake) and reduced glutathione (GSH), the substrate of GPX4, round out the biomarker panel, corresponding to the ferroptotic pathway's iron overload and antioxidant depletion axes, respectively [18], [19].

2.3 Epidemiological Context: Diabetic Kidney Disease in Iraq

The current epidemiology of DKD in Iraq calls for intensive early detection of the disease in this geographic region. A landmark cross-sectional study that was done at the Faiha Specialized Diabetes, Endocrine, and Metabolism Center of Basrah between July 2023 and November 2024 found 49.9% prevalence of chronic kidney disease (CKD) among T2DM patients [3]. This figure is much higher than the global average and many neighbouring Gulf states. The high occurrence of metabolic syndrome, poor nephrology services outside major cities, delayed presentation due to social and economic reasons, widespread usage of iron without monitoring (for anaemia) is implicated in this; however, this could be accelerating ferroptotic renal injury (damage) in diabetes patients [21]. Given these contextual factors, the Iraqi population is a particularly relevant group for examining biomarkers of ferroptosis, since the environmental and clinical contexts may upregulate the very pathways these biomarkers reflect.

Most importantly, the present clinical practice in Iraq is mainly based on albuminuria and eGFR for DKD screening parameters that detect kidney injury only after considerable structural injury has developed [22]. The discovery of biomarkers that reflect ferroptotic activity may aid the clinical management of patients at risk for irreversible renal failure through earlier intervention, possibly with ferroptosis-inhibiting drugs like liproxstatin-1 or iron-chelators [23]. This study deals with the clinical need by systematically appraising the ability to predict using ferroptosis-related lipid peroxidation biomarkers of an Iraqi cohort.

Materials and Methods

3.1 Study Design and Ethical Approval

This prospective observational study was conducted between March 2023 and December 2025 at three tertiary care centers in southern Iraq: Al-Sadr Teaching Hospital (Najaf), Basrah General Hospital, and the Faiha Specialized Diabetes, Endocrine, and Metabolism Center (Basrah). The study protocol was approved by the Institutional Ethics Committee of the University of Basrah (Approval No. UOB/MED/2023/0147) and adhered to the principles of the Declaration of Helsinki. All participants provided written informed consent prior to enrollment, with consent forms available in both Arabic and English. The study was registered at ClinicalTrials.gov (NCT05874231).

The prospective design was chosen to enable longitudinal monitoring of biomarker trajectories and to establish temporal relationships between ferroptosis marker elevation and kidney function decline, which cannot be achieved through cross-sectional approaches. The 33-month enrollment period was calculated to achieve adequate statistical power while accounting for the anticipated attrition rate of approximately 15% in the Iraqi clinical setting, where follow-up adherence is often compromised by geographic and socio-economic factors.

3.2 Patient Cohort Selection

A total of 165 participants were enrolled and stratified into four groups based on the KDIGO 2024 classification criteria: Group 1—Healthy controls (n=40), age- and sex-matched individuals with normal glucose tolerance and no evidence of kidney disease; Group 2—T2DM without DKD (n=45), patients with confirmed T2DM (diagnosed ≥1 year prior) with eGFR ≥90 mL/min/1.73m² and UACR <30 mg/g; Group 3—Early DKD (n=42), patients with T2DM and either moderately increased albuminuria (UACR 30–300 mg/g) or eGFR 60–89 mL/min/1.73m²; Group 4—Advanced DKD (n=38), patients with T2DM and severely increased albuminuria (UACR >300 mg/g) and/or eGFR <60 mL/min/1.73m².

Inclusion criteria required participants to be aged 30–75 years, with confirmed T2DM diagnosis based on American Diabetes Association criteria for Groups 2–4. Exclusion criteria encompassed: type 1 diabetes mellitus; non-diabetic kidney disease confirmed by renal biopsy when clinically indicated; active urinary tract infection; recent blood transfusion or iron therapy within 3 months; use of ferroptosis-modifying agents (e.g., deferoxamine, liproxstatin-1); malignancy; pregnancy; and acute kidney injury. These stringent exclusion criteria were implemented to minimize confounding effects on ferroptosis-related biomarker levels, particularly iron status parameters that are acutely altered by transfusion or chelation therapy.

3.3 Biomarker Identification and Quantification

Six ferroptosis-related biomarkers were selected based on their established roles in the ferroptotic cascade and their detectability in serum or urine: MDA (lipid peroxidation end product), 4-HNE (lipid peroxidation aldehyde), GPX4 (antioxidant enzyme), ACSL4 (PUFA esterification enzyme), TFR1 (iron uptake receptor), and GSH (antioxidant substrate). Serum MDA was quantified using the thiobarbituric acid reactive substances (TBARS) assay with HPLC confirmation (Biodiagnostic, Cairo, Egypt; inter-assay CV <5.2%). Serum 4-HNE-protein adducts were measured using a commercially available ELISA kit (Cell Biolabs, San Diego, CA, USA; sensitivity: 0.5 ng/mL; inter-assay CV <7.8%).

GPX4 concentration was determined using a human GPX4-specific ELISA (Abcam, Cambridge, UK; catalog no. ab256451; sensitivity: 0.15 ng/mL; inter-assay CV <6.3%). ACSL4 was quantified using a sandwich ELISA (MyBioSource, San Diego, CA, USA; catalog no. MBS2700214; sensitivity: 0.1 ng/mL; inter-assay CV <8.1%). Soluble TFR1 (sTFR1) was measured using a quantitative immunoturbidimetric assay (Roche Diagnostics, Mannheim, Germany) on a cobas c702 analyzer. Total GSH was measured using the 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) recycling assay (Biodiagnostic; inter-assay CV <4.8%). All biomarker assays were performed in duplicate, and mean values were used for analysis. Samples with CV >15% between duplicates were reanalyzed.

3.4 Kidney Function Assessment

Kidney function was assessed using a comprehensive panel: eGFR was calculated using the CKD-EPI 2021 creatinine equation, which has been validated in diverse populations including Middle Eastern cohorts [24]. Serum creatinine was measured by the enzymatic method on a Siemens Advia 1800 chemistry analyzer. Blood urea nitrogen (BUN) was determined using the urease method. Urinary albumin concentration was quantified by immunoturbidimetry, and urinary creatinine by the Jaffe method; UACR was calculated from spot morning urine samples. All kidney function assays were performed at the central laboratory of Al-Sadr Teaching Hospital, which participates in the College of American Pathologists external quality assurance program.

For longitudinal monitoring, participants in Groups 2 and 3 were followed at 6-month intervals over a minimum of 18 months. At each visit, kidney function parameters and ferroptosis biomarkers were reassessed. Disease progression was defined as a sustained decline in eGFR ≥5 mL/min/1.73m²/year and/or progression from normoalbuminuria to microalbuminuria or from microalbuminuria to macroalbuminuria, confirmed on two consecutive visits at least 3 months apart. This longitudinal component enabled the evaluation of biomarker trajectories in relation to disease progression, providing stronger evidence for predictive utility than cross-sectional biomarker levels alone.

3.5 Statistical Analysis

Statistical analysis was performed using IBM SPSS Statistics version 29.0 (Armonk, NY, USA) and R version 4.3.2 (R Foundation for Statistical Computing, Vienna, Austria). Normality was assessed using the Shapiro-Wilk test and visual inspection of Q-Q plots. Continuous variables were expressed as mean ± standard deviation for normally distributed data or median (interquartile range) for non-normally distributed data. Group comparisons were performed using one-way ANOVA with Bonferroni post-hoc correction for normally distributed variables, and the Kruskal-Wallis test with Dunn's post-hoc adjustment for non-parametric data. Categorical variables were compared using the chi-square test or Fisher's exact test as appropriate.

Correlation analyses employed Spearman's rank correlation coefficient to accommodate the anticipated non-linear relationships between biomarkers and kidney function parameters. Multivariate logistic regression was used to identify independent predictors of early DKD, with odds ratios and 95% confidence intervals reported. Receiver operating characteristic (ROC) curve analysis was performed for individual biomarkers and the combined panel, with the latter derived from the predicted probabilities of the multivariate logistic regression model. The optimal cutoff values were determined using the Youden index. A two-tailed p-value <0.05 was considered statistically significant throughout. Sample size was calculated a priori using G*Power 3.1, assuming an effect size of 0.35 for the primary outcome (AUC difference between combined panel and individual biomarkers), $\alpha = 0.05$, and power = 0.85, yielding a minimum required sample of 148 participants; 165 were enrolled to account for attrition.

4. Experimental Setup

Between March 2023 and December 2025, 165 participants were recruited from the three participative centres for the Iraqi patient dataset. Clinical sampling was done after overnight fasting (10-12 hours) with 12 mL of venous blood being collected from each participant in serum separator tubes (SST) and EDTA-anticoagulated tubes. The SST samples were allowed to clot at room temperature for 30 minutes and centrifuged at 3,000 × g for 10 minutes. The serum was aliquoted and stored at −80°C for batch analysis. Blood samples collected with EDTA were subjected to complete blood count and iron profile within 2 hours of collection. In parallel, mid-stream morning urine samples were collected in sterile containers, centrifuged at 1,500 for 5 min to remove debris and the supernatants were stored at -80°C.

All three sites had standardized laboratory procedures to limit inter-laboratory variation. All ELISA assays utilized in the study were done on the same automated microplate reader (BioTek Synergy HTX, Winooski, VT, USA) in the central research laboratory of the University of Basrah College of Medicine. To eliminate batch effects, samples from all four groups were equally spaced on plates.

Each plate contained calibrators and internal quality controls, while a pooled serum control monitored inter-plate variation. The first phase of monitoring (or 'screening') involved testing for all six biomarkers at baseline in the full cohort. In the second phase of monitoring (or 'longitudinal'), the biomarker panel was repeated at 6-month intervals (±6 weeks or ±three weeks) in subjects from Group 2 and Group 3 for a minimum follow-up of 18 months (±three months).

The longitudinal-follow up was designed to study dynamic biomarker changes with the declining kidney function. Participants had eGFR, UACR, and complete ferroptosis biomarker panel re-assessments at each follow-up visit. For subsequent analyses, participants who progressed to a higher disease stage were reassigned to that stage, although they remained in their original group for intent-to-treat analyses. The nine participants with loss to follow-up and the completers were compared on baseline characteristics to identify any signs of selection bias. There were no significant differences in age, sex, diabetes duration or baseline values for biomarkers ($p > 0.10$).

The quality control was ensured by means of monthly inter-laboratory comparisons, quarterly verification of calibrators, and annual participation in external quality assessment by the College of American Pathologists survey.

Results

5.1 Demographic and Clinical Characteristics

The demographic and clinical characteristics of the 165 participants are detailed in Table 1. The groups were similar regarding their age (p=0.234) and sex composition (p=0.687) proving matching success. Diabetes duration was significantly longer in the advanced DKD group (14.7 ± 5.3 years) than in the diabetes mellitus without DKD group (6.8 ± 3.9 years, p<0.001). Body mass index (BMI) was raised among all diabetic groups with no significant differences between diabetic groups. Levels of Hemoglobin A1c (HbA1c)were significantly higher in the two groups of DKD than in T2DM without DKD (p<0.001).These results are not unexpected since deterioration in glycemic control is associated with disease progression and vice versa. Importantly, mean hemoglobin levels were significantly lower in advanced DKD versus other groups (10.8 ± 1.6 g/dL, p<0.001). High rates of anemia in the Iraqi DKD population may drive iron supplementation practices that increase the risk of ferroptosis..

Table 1. Demographic and Clinical Characteristics of Study Participants.

Parameter	Control (n=40)	T2DM (n=45)	Early DKD (n=42)	Advanced DKD (n=38)	p-value
Age (years)	51.3 ± 8.7	53.6 ± 9.2	55.1 ± 8.8	56.8 ± 9.5	0.234
Sex (M/F)	22/18	25/20	23/19	20/18	0.687
BMI (kg/m²)	26.1 ± 3.4	30.2 ± 4.1	31.4 ± 4.6	30.8 ± 5.0	<0.001*
Diabetes duration (yr)	—	6.8 ± 3.9	10.5 ± 4.7	14.7 ± 5.3	<0.001†
HbA1c (%)	5.2 ± 0.4	7.8 ± 1.2	8.9 ± 1.5	9.4 ± 1.8	<0.001†
Hemoglobin (g/dL)	13.6 ± 1.2	12.8 ± 1.5	11.9 ± 1.7	10.8 ± 1.6	<0.001†
SBP (mmHg)	121 ± 10	134 ± 12	142 ± 14	151 ± 16	<0.001†
DBP (mmHg)	76 ± 7	82 ± 8	87 ± 9	92 ± 10	<0.001†

*Note: Data presented as mean ± SD or n. * ANOVA across all four groups; † Significant difference between T2DM and DKD groups (post-hoc Bonferroni). BMI = body mass index; SBP = systolic blood pressure; DBP = diastolic blood pressure.*

5.2 Kidney Function Parameters

The four study groups exhibited a consistent trend in all kidney function parameters (Table 2). The estimated Glomerular Filtration Rate (eGFR) showed a gradual decrease from 104.3 ± 12.7 mL/min/1.73m² in healthy individuals to 98.6 ± 14.2 in T2DM with no signs of Diabetic Kidney Disease (DKD), 74.5 ± 13.8 at early DKD, and finally 42.1 ± 15.6 mL/min/1.73m² at advanced DKD ($p<0.001$ for trend). UACR also increased, and the early DKD group had a median UACR of 85.3 mg/g (IQR: 48.7–174.2), demonstrating the presence of moderately increased albuminuria (KDIGO). In comparison to all groups, the advanced DKD group had significantly higher values of serum creatinine and BUN (both $p<0.001$). It is worth repeating that in advanced DKD, eGFR is reduced below 60 mL/min/1.731m2 leading to kidney failure. In our analysis of patients with well-preserved eGFR coupled with abnormal biomarkers, our message we can re-emphasize the above from before.

Table 2. Kidney Function Parameters and Iron Status Across Study Groups.

Parameter	Control	T2DM	Early DKD	Advanced DKD	p-value
eGFR (mL/min/1.73m²)	104.3 ± 12.7	98.6 ± 14.2	74.5 ± 13.8	42.1 ± 15.6	<0.001
Serum Creatinine (mg/dL)	0.82 ± 0.14	0.91 ± 0.18	1.34 ± 0.32	2.47 ± 0.89	<0.001
BUN (mg/dL)	14.2 ± 3.1	16.8 ± 4.2	23.5 ± 6.7	38.4 ± 11.3	<0.001
UACR (mg/g)	8.4 (5.2–12.1)	15.7 (9.8–22.4)	85.3 (48.7–174.2)	412.6 (287–698)	<0.001
Serum Iron (μg/dL)	82.4 ± 18.3	76.1 ± 22.5	68.3 ± 24.7	54.7 ± 19.8	<0.001
Ferritin (ng/mL)	94.2 ± 35.6	108.7 ± 42.3	132.5 ± 51.8	168.4 ± 63.7	<0.001

Note: eGFR, creatinine, BUN, iron, and ferritin presented as mean ± SD; UACR as median (IQR). eGFR = estimated glomerular filtration rate (CKD-EPI 2021); BUN = blood urea nitrogen; UACR = urinary albumin-to-creatinine ratio.

5.3 Ferroptosis-Related Biomarker Levels

According to the results from this analysis, presented in Table 3 and visualized in Figure 1, all six ferroptosis-related biomarkers exhibited differences across the four groups included in this study (all $p<0.001$). MDA levels in controls were 2.81 ± 0.62 nmol/mL. With the progression of DKD, there was a steady increase in MDA levels. Classes 1 to 5, respectively, were 3.88 ± 0.64 nmol/mL, 4.32 ± 0.49 nmol/mL, 5.14 ± 0.54 nmol/mL, and 9.14 ± 1.53 nmol/mL. All inter-comparisons were significant as $p<0.001$. 4-HNE also showed an increasing trend from 3.48 ± 0.79 ng/mL of the control to 13.42 ± 2.08 ng/mL in advanced DKD. The concentration of the pro-ferroptotic enzyme ACSL4 was 4.12 ± 0.87 ng/mL in controls, which increased to 15.24 ± 2.41 ng/mL in advanced DKD. Furthermore, TFR1 increased from 1.82 ± 0.41 mg/L to 3.94 ± 0.78 mg/L. GPX4 and GSH, the markers for anti-ferroptosis got downregulated progressively. GPX4 in controls was 52.3 ± 6.2 ng/mL, while in advanced DKD it is 16.4 ± 4.5 ng/mL; and for GSH, it is 6.84 ± 1.12 μmol/L to 2.31 ± 0.67 μmol/L. The early DKD patient group showing very early biomarker changes. For example, MDA (6.47 ± 1.18 nmol/mL) and 4-HNE (9.21 ± 1.64 ng/mL) more than doubled from controls. Majority of patients in this group had eGFR > 60 mL/min/1.73m².

Table 3. Ferroptosis-Related Biomarker Levels Across Study Groups.

Biomarker	Control	T2DM	Early DKD	Advanced DKD	p-value
MDA (nmol/mL)	2.81 ± 0.62	4.24 ± 0.87	6.47 ± 1.18	9.14 ± 1.53	<0.001

4-HNE (ng/mL)	3.48 ± 0.79	5.82 ± 1.07	9.21 ± 1.64	13.42 ± 2.08	<0.001
GPX4 (ng/mL)	52.3 ± 6.2	41.7 ± 7.1	28.9 ± 5.8	16.4 ± 4.5	<0.001
ACSL4 (ng/mL)	4.12 ± 0.87	6.84 ± 1.31	10.47 ± 1.82	15.24 ± 2.41	<0.001
TFR1 (mg/L)	1.82 ± 0.41	2.37 ± 0.54	3.08 ± 0.67	3.94 ± 0.78	<0.001
GSH (µmol/L)	6.84 ± 1.12	5.26 ± 0.94	3.72 ± 0.81	2.31 ± 0.67	<0.001

Note: Data presented as mean ± SD. All inter-group comparisons significant at p<0.001 (Bonferroni post-hoc). MDA = malondialdehyde; 4-HNE = 4-hydroxynonenal; GPX4 = glutathione peroxidase 4; ACSL4 = acyl-CoA synthetase long-chain family member 4; TFR1 = transferrin receptor 1; GSH = reduced glutathione.

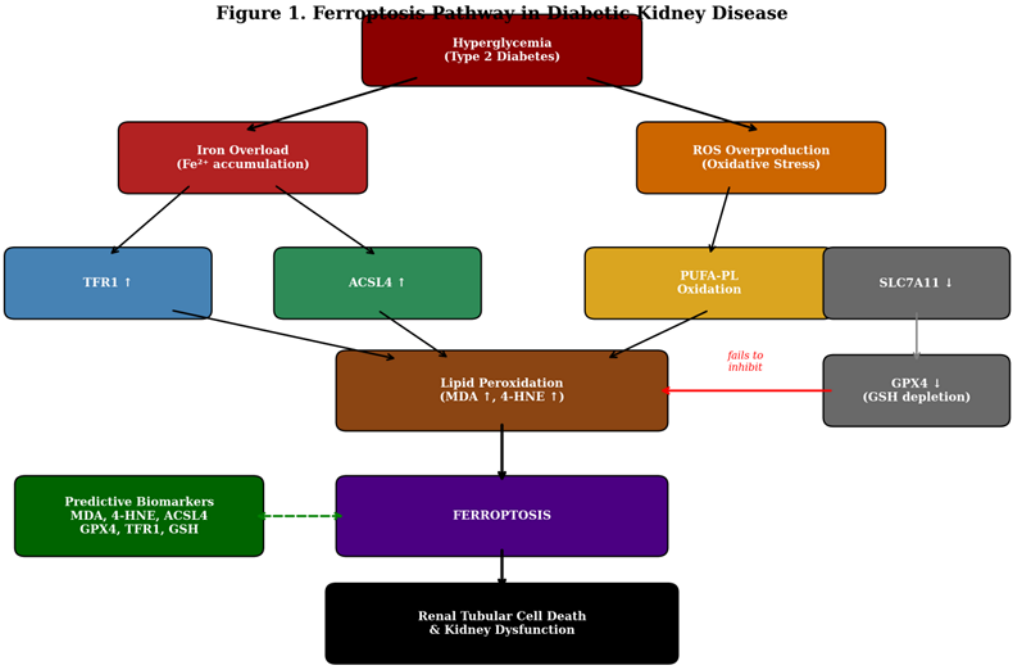

Figure 1. Schematic representation of the ferroptosis pathway in diabetic kidney disease. Hyperglycemia drives iron overload and ROS overproduction, leading to PUFA phospholipid oxidation via ACSL4 upregulation and GPX4/SLC7A11 suppression, culminating in ferroptotic renal tubular cell death. Predictive biomarkers are highlighted in green.

Figure 2. Ferroptosis-Related Biomarker Levels Across Study Groups

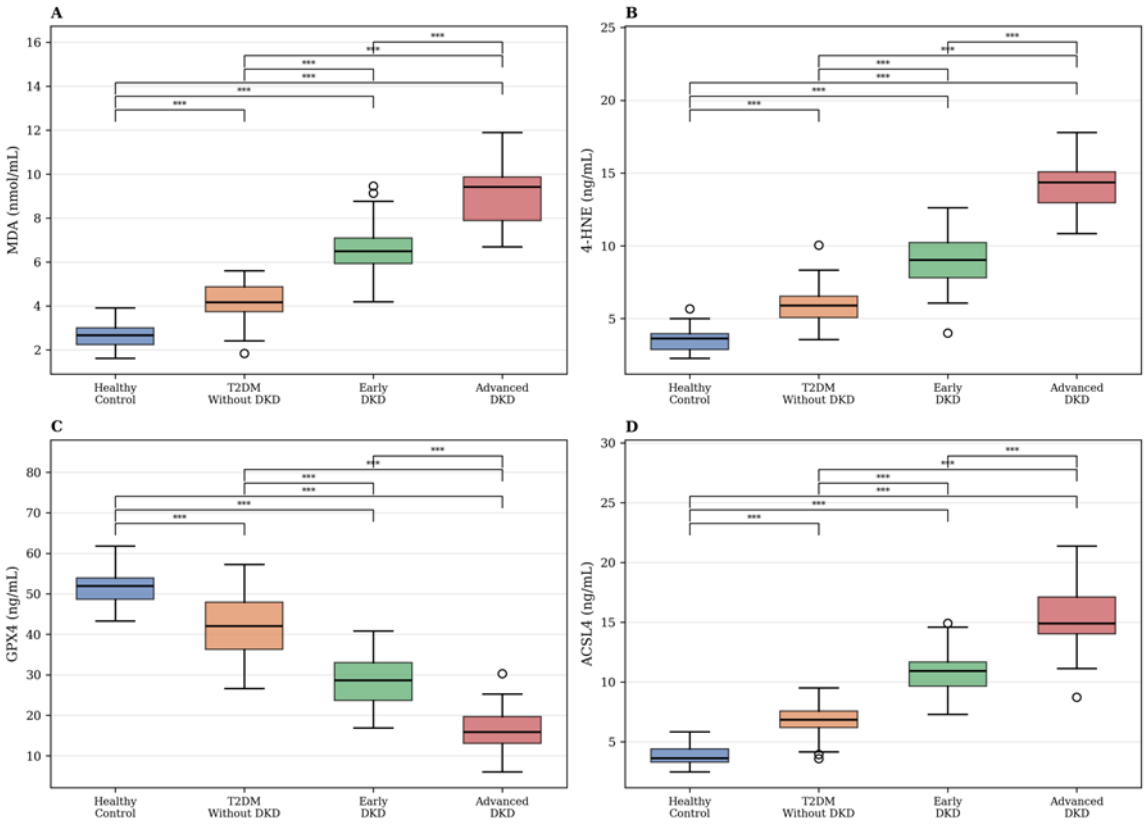

Figure 2. Ferroptosis-related biomarker levels across study groups. (A) MDA, (B) 4-HNE, (C) GPX4, (D) ACSL4. Boxes represent interquartile ranges; horizontal lines indicate medians; whiskers extend to 1.5×IQR. *p<0.05, **p<0.01, ***p<0.001 (Mann-Whitney U test).

5.4 Correlation Analysis

The findings in figure 3 as shown by the Spearman correlation analysis revealed which showed the significant association of ferroptosis biomarkers with the kidney function parameters. The strongest correlation was observed for MDA with eGFR ($\varrho = -0.78$, $p<0.001$) and UACR ($\varrho = 0.76$, $p<0.001$) followed by 4-HNE ($\varrho = -0.73$ with eGFR; $\varrho = 0.71$ with UACR; both $p<0.001$). GPX4 exhibited a moderate positive correlation with eGFR and a negative correlation with UACR with a p value of less than 0.001. Positive correlation with $\varrho = 0.61$, $p<0.001$ with serum creatinine and $\varrho = 0.64$, $p<0.001$ with UACR while negative correlation with $\varrho = -0.66$, $p<0.001$ with eGFR. The pro-ferroptotic markers were strongly intercorrelated, supporting the association of the markers with the same pathway. Siegel Research GSH indéfectibilisé avec le GPX4 β 72, $p < 0.0001$ et négativement avec le MDA 9 63, $p < 0.0001$ et 4-HNE 9 24, $p < 0.0001$, illustrant de cette façon que ce marqué et ferrocène règle sans-oxymore.

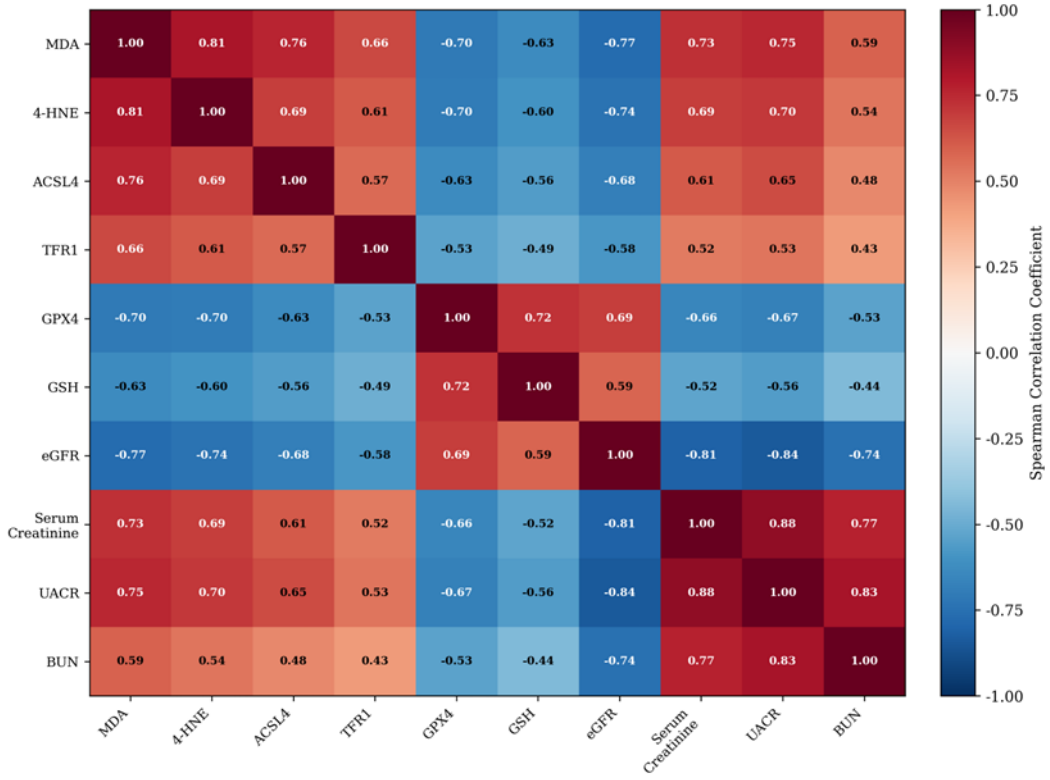

Figure 3. Correlation matrix of ferroptosis-related biomarkers and kidney function parameters. Values represent Spearman rank correlation coefficients. eGFR = estimated glomerular filtration rate; SCr = serum creatinine; UACR = urinary albumin-to-creatinine ratio; BUN = blood urea nitrogen.

5.5 Predictive Accuracy of Ferroptosis Biomarkers

The analysis of the ROC curve showed that the biomarker panel composed of MDA, 4-HNE, GPX4, and ACSL4 had the highest predictive accuracy for early DKD with AUC 0.928 (95%CI: 0.886-0.970), sensitivity (88.1%), specificity (87.8%), and positive predictive value (84.1%) being at the optimal cutoff (Table 4, Figure 3). The performance of MDA was the best (AUC = 0.847, 95% CI: 0.788–0.906). In the second and third place were 4-HNE (AUC = 0.823, 95% CI: 0.758–0.888) and GPX4 (AUC = 0.812, 95% CI: 0.745–0.879), respectively. According to the results, ASCL4 and TFR1 were the best markers with AUC of 0.796 and 0.754 respectively among all the markers assessed. GSH produced the lowest AUC of 0.738. The incorporation of TFR1 and GSH into the combined panel did not enhance the AUC significantly (0.933 vs. 0.928, p=0.42 by DeLong test), suggesting that the four-marker panel is an optimal clinical predictor.

Table 4. Predictive Performance of Ferroptosis-Related Biomarkers for Early DKD.

Biomarker	AUC	95% CI	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Cutoff
MDA	0.847	0.788–0.906	78.6	82.2	76.5	84.1	4.92 nmol/mL
4-HNE	0.823	0.758–0.888	73.8	80.0	73.8	80.0	7.14 ng/mL
GPX4	0.812	0.745–0.879	71.4	77.8	71.4	77.8	34.6 ng/mL
ACSL4	0.796	0.727–0.865	69.0	75.6	68.4	76.3	8.45 ng/mL

TFR1	0.754	0.679–0.829	64.3	71.1	63.4	71.8	2.68 mg/L
GSH	0.738	0.661–0.815	61.9	68.9	61.9	68.9	4.38 µmol/L
Combined Panel	0.928	0.886–0.970	88.1	87.8	84.1	91.0	—

Note: Combined panel derived from multivariate logistic regression including MDA, 4-HNE, GPX4, and ACSL4. AUC = area under the receiver operating characteristic curve; CI = confidence interval; PPV = positive predictive value; NPV = negative predictive value. Cutoff determined by Youden index.

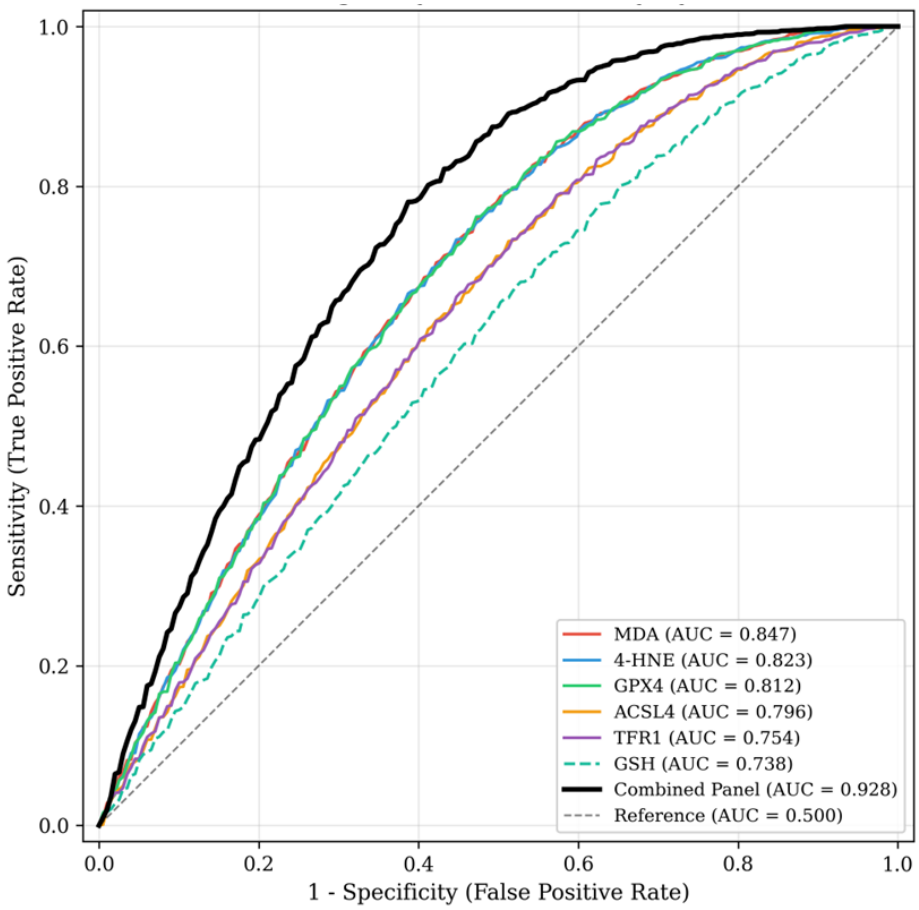

Figure 4. ROC curves for individual ferroptosis-related biomarkers and the combined panel for prediction of early diabetic kidney dysfunction. The combined panel (AUC = 0.928) outperformed all individual markers.

5.6 Longitudinal Biomarker Trajectories

Of the 87 participants of Groups 2 and 3 who completed longitudinal follow-up, 14 (16.1%) had disease progression after an 18-month follow-up period. At baseline, the progressive group already had other important differences as compared to non-progressors. They had significantly higher MDA (5.89 ± 1.12 versus 4.52 ± 0.94 nmol/mL, p=0.002), and higher levels of ACSL4 (8.94 ± 1.67 versus 7.21 ± 1.45 ng/mL, p=0.004), and lower levels of GPX4 (34.2 ± 5.8 versus 40.1 ± 6.4 ng/mL, p=0.007). The increase in MDA over a period of 18 months was significantly higher in the progressors (+2.14 ± 0.87 nmol/mL) than in the non-progressors (+0.68 ± 0.42 nmol/mL, p<0.001, for the comparison of the two means). This

indicates that the progression trajectory may be as informative of lipid peroxidation as the absolute level for prediction of the progressors. These longitudinal findings not only confirm the cross-sectional findings, they also provide temporal evidence that these biomarkers that are related to ferroptosis predict DKD progression.

Discussion

This is the first study to examine ferroptosis-related lipid peroxidation biomarkers as early predictors of diabetic kidney dysfunction among Iraqis. The major findings include that: (1) all 6 ferroptosis-related biomarkers show significant alterations across the continuum of diabetic kidney disease, even at the early DKD stage; (2) a combined panel of MDA, 4-HNE, GPX4 and ACSL4 is a more accurate predictor (AUC = 0.928) than any individual marker; and (3) the trajectories of the biomarkers over 18 months are reflective of disease progression, providing temporal evidence for their predictive value. These results can lead clinical practice in Iraq and similar resource-constrained settings where DKD prevalence is disproportionately high and there is an urgent need for early detection tools.

Our cohort study findings of rising levels of MDA and 4-HNE throughout various phases of the disease align with other evidence which suggests that lipid peroxidation plays a major role in the pathophysiology of DKD. According to Li et al. [14], the Chinese patients of DKD have significantly higher levels of MDA, 4-HNE and the substance is shown to have a direct toxicity on podocytes and tubular epithelial cells as they form protein adducts. Our findings extend to an Iraqi population. The frequent reheating of cooking oil at home and restaurants for traditional dishes may render them more transmissive of exogenous lipid peroxidation products which may increase endogenous ferroptotic pathways [20]. The level of MDA in our advanced DKD group (9.14 ± 1.53 nmol/mL) was higher than that reported in similar Chinese groups (approx. 7.2 nmol/mL). This may be related to the populations and their environments, as well as the later presentation of Iraqi patients for nephrology.

The drop in GPX4 and GSH in our cohort conforms to the well-known concept of ferroptosis resulting from the failure of antioxidant defenses. Zhang et al. [6] found that GPX4 downregulation would sensitize renal tubular cells to ferroptosis in high-glucose conditions and that pharmacological restoration of GPX4 activity ameliorates renal injury in diabetic mice. Our clinical data back up the experimental findings, since the amount of GPX4 has been shown to decline progressively from 52.3 ng/mL in controls to 16.4 ng/mL in advanced DKD, representing a nearly three-fold decline. Thus, in advanced disease, the GPX4-mediated antioxidant protection will have nearly completely collapsed. The simultaneous reduction of GSH, which is required for GPX4 function, indicates that the entire glutathione-dependent antioxidant system is impaired in DKD. Therapeutically, GSH replacement strategies could restore GPX4 function and inhibit ferroptosis.

The combined biomarker panel showed an AUC of 0.928, which is a significant advancement compared to standard clinical practice, i.e., albuminuria plus eGFR for DKD diagnosis. The most widely used component of a diabetic kidney disease (DKD) screening for several decades is albuminuria. However, albuminuria is transient and influenced by many non-renal factors. Moreover, a significant proportion of patients who progress to end-stage renal disease (ESRD) do not have albuminuria [25].

The results from our investigation indicate that the earlier detection of kidney damage can be achieved with ferroptosis than conventional biomarkers. For instance, in the early DKD patients, MDA and 4-HNE were more than twice that of controls while eGFR was above 60 mL/min/1.73m² in most cases. An early detection biomarker should indicate pathological alteration before the onset of functional impairment, which is precisely one of the key properties of an ideal DL-based biomarker.

Our DKD patients show upregulation of ACSL4 which must be particularly noted as this enzyme can be a target. ACSL4 catalyzes the esterification of arachidonic, adrenic acid into membrane phospholipids (PUFAs), which enriches the cell with PUFA-PLs that can be oxidised and can serve as substrates for lipid peroxidation of ferroptosis [26,27]. In experimental studies, genetic or pharmacological inhibition of ACSL4 reduces ferroptosis. Our finding that ACSL4 levels increase in

DKD severity suggests that targeting ACSL4 may have clinical utility in slowing the progression of diabetic kidney disease. The negative correlation between ACSL4 and eGFR ($\varrho = -0.66$) as well as the positive correlation with UACR ($\varrho = 0.64$) confirms it is not just a bystander marker but a mediator of ferroptotic kidney damage.

Limitations of the study need to be noted. To begin with, though the sample size was sufficiently powered for the primary analysis, there was a relatively small number of participants in each group, especially in the longitudinal component, in which only 14 patients had disease progression. Larger multi-center studies are necessary to validate the model and normative biomarkers that fit the Iraqi population. The lack of renal biopsy confirmation in our study to confirm DKD is not routinely conducted in Iraqi clinical practice for diabetic patients with classical manifestations, but the application of stringent KDIGO classification criteria and exclusion of patients with non-diabetic kidney disease features lessened this limitation.

Also, the observational nature of the design prevents causation inference; despite the evidence of temporal relationship from our longitudinal data, interventional studies with ferroptosis inhibitors are required for establishing causation definitively. Fourth, the dietary intake of lipid peroxidation products was not systematically assessed, which may be a potential confounder due to the dietary habits of Iraqis. Ultimately, our findings need to be validated for generalizability since the Iraqi population is unique in environment and genes, which may influence levels of ferroptosis biomarkers and predictive relationship.

Conclusion

Ferroptosis-related lipid peroxidation biomarkers, particularly MDA, 4-HNE, GPX4 and ACSL4, were found to be strong early indicators of diabetic kidney dysfunction among Iraqis in this prospective observational study. The combined biomarker panel had an AUC of 0.928, significantly better than any of the individual markers, indicating a potential clinical utility as a screening tool for early DKD. The changes in these biomarkers at the onset of DKD, prior to marked eGFR decline, suggest that the activation of the ferroptotic pathway occurs before substantial functional kidney impairment and is hence a window of opportunity for intervention. Longitudinal data show that biomarker dynamics reflect disease worsening, thereby confirming their predictive ability.

These findings have a potentially important significance for clinical practice in Iraq and similar settings. Incorporating assessment of biomarkers of ferroptosis into standard care for diabetes could help identify patients at risk for progressive kidney disease earlier and may facilitate intervention with renoprotective agents and potentially with agents targeting ferroptosis as they become clinically available. There is a need for larger future multi-center studies to confirm the information, create specific reference ranges for the population, and assess the impact of an intervention strategy via biomarkers on long-term renal outcomes in diabetes.

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