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The Role of Vitamin D, DKK1, Hepcidin and Oxidative Stress Biomarkers in Type 2 Diabetes Mellitus Patients With and Without Diabetic Nephropathy

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ABSTRACT

Background: Type 2 diabetes mellitus (T2DM) is a widespread disease. Diabetic nephropathy (DN) is one of the most prevalent and harmful effects of T2DM.

Objectives: We aimed to assess blood levels of vitamin D, dickkopf1 (DKK1), hepcidin, and oxidative stress biomarkers in T2DM patients who have and do not have DN.

Subjects and methods: The study comprised 55 T2DM patients, of which 35 had DN, 20 did not, and 30 were healthy controls. ELISA was utilized to estimate serum concentrations of vitamin D, DKK1, and hepcidin, while spectrophotometry was used to detect the oxidative stress indicators.

Results: Comparing T2DM patients to controls and DN patients to patients without DN, serum levels of DKK1, hepcidin, lipid peroxide (LPER), and nitric oxide (NO) were considerably greater, whereas vitamin D, glutathione peroxidase (GPx), and superoxide dismutase (SOD) were significantly lower. DKK1, hepcidin, LPER, and NO levels were considerably higher in T2DM patients with prolonged duration and inadequate glycemic control, but vitamin D, GPx, and SOD levels were significantly lower. Vitamin D, DKK1, and SOD showed the highest predictive value for T2DM. Vitamin D and hepcidin, meanwhile, demonstrated the strongest predictive value for DN. In T2DM patients, elevated hepcidin levels were strong predictor of DN. Vitamin D correlated positively with GPx and SOD and negatively with DKK1, hepcidin, LPER and NO.

Conclusion: We can deduce that in T2DM patients, especially those with DN, long duration, and poor glycemic control, high levels of DKK1, hepcidin, LPER, NO, and low concentrations of vitamin D, GPx, and SOD are detected. Hepcidin may be useful in diagnosis and predicting DN in T2DM patients. This emphasizes the role of these biomarkers in pathogeneses of T2DM and DN in an effort to have potential therapeutic implications in the future.

Keywords: Type 2 diabetes mellitus, Diabetic nephropathy, Vitamin D, DKK1, Hepcidin, Oxidative stress.

INTRODUCTION

Diabetes mellitus (DM) is a metabolic disease marked by hyperglycemia brought on by deviations in insulin effect, synthesis, or both. T2DM is the most common type of diabetes, makes up for 90% of diabetes [(1)]. One of the most prevalent and harmful effects of DM is DN. It is the primary contributor to end-stage renal disease (ESRD) [(2)]. It may be advantageous to use biomarkers that are indicative of the incidence and progression of T2DM and its consequences in order to diagnose and treat patients earlier in the course of the illness [(3)].Having hormonal effects, vitamin D is a fat-soluble vitamin. Reactive oxygen species (ROS) and Ca^{2+} levels are maintained at normal resting levels by vitamin D in both insulin-responsive tissues and pancreatic β-cells [(4)]. The body's sensitivity to insulin is thought to be enhanced by vitamin D, which lowers the likelihood of developing insulin resistance. However, the connection between vitamin D levels and T2DM is unknown. A serious global public health problem is vitamin D deficiency. It substantially contributes to the development of T2DM [(5)].

Dickkopf1 (DKK1) is a small molecular weight soluble secreted protein. DKK1 is a powerful inhibitor of Wnt signalling and implicated in the control of glucose metabolism [(6)]. In T2DM, circulating DKK1 is elevated. Dysfunction of podocyte and mesangial cell is a factor in DN. The relevance of dysregulated DKK1/Wnt/β-catenin signalling pathways in the pathophysiology of glomerular diabetic lesions is being increasingly supported by research [(7)].

Hepcidin is the principal iron regulator. The liver increases hepcidin synthesis in response to increased iron levels, and this action on the sites of absorption, storage, or recycling causes a reduction in the release of iron from these tissues [(8)]. Erythropoiesis, inflammation, and levels of both circulating and stored iron all influence hepcidin levels [(9)]. Hepcidin may have a role in the aetiopathogenesis of T2DM because T2DM is one of the consequences of having too much iron in the body. Excessive systemic iron can damage hepatocytes and pancreatic β cells owing to oxidative stress [(10)].

Oxidative stress (OS) is imbalance between the production of reactive oxygen species (ROS) and antioxidant defence mechanisms [(3)]. OS has been regarded as a key indicator of the pathophysiology and occurrence of T2DM and related consequences [(11)]. Pancreatic β-cells experience oxidative damage as a result of chronic hyperglycemia. ROS are capable of causing DNA, protein, and lipid damage, which results in β-cell malfunction and death [(3)].

Given the foregoing, the current research was carried out to assess the serum levels of vitamin D, DKK1, hepcidin, and biomarkers of oxidative stress in T2DM patients and to examine their relationship with DN with the purpose of better grasping their potential

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roles in the pathogenesis and future management of this severe complication. We also wanted to find out how these biomarkers were affected by glycemic control and duration of T2DM.

SUBJECTS AND METHODS

1. Study subjects

On 55 T2DM patients, the current case-control research was undertaken. The samples were collected from the Outpatient Clinic and Inpatients of the Internal Medicine Department of Assiut University Hospital, Egypt, between September 2020 and August 2021. Patients were divided into 25 without DN and 30 T2DM patients with DN. Additionally, 30 people in perfect health who served as the control group were comprised in the research. By measuring the urine albumin to creatinine ratio (UACR) in a spot urine sample, diabetic nephropathy was identified. Values >30 mg/mg creatinine were interpreted as nephropathy. Other forms of diabetes, a recent history of an acute illness, chronic liver or renal disease, pregnancy, breastfeeding, a history of cancer, malnutrition, hyperparathyroidism or hypoparathyroidism, nephrolithiasis, and the use of any medications known to affect vitamin D levels, such as multivitamins, anticonvulsants, glucocorticoids, and rifampin, were all considered exclusion criteria. Every patient had a full medical history assessment and physical examination.

2. Ethical considerations

Assiut University Faculty of Medicine's Ethics Committee gave its approval for this study (IRB no. 17300792), which was carried out in accordance with the guidelines outlined in the Declaration of Helsinki. All study participants gave their informed consent.

3. Sample collection

Five millilitres of blood were drawn from each participant after an overnight fast, and the samples were split into two containers: one with EDTA for the HbA1c and Hb assay, and the other without the anticoagulant, which was left for 30 minutes at room temperature before being centrifuged for 20 minutes at 3000 rpm. The serum from the supernatant was collected, divided into aliquots, and some of it was used instantly to estimate glucose. The remainder was stored at -80°C until it was time to analyze the other examined parameters. The hemolyzed samples were thrown away. A mid-stream urine sample was collected in the early morning. Before usage, the cloudy samples were centrifuged and the clear floating segment was held at -20°C to determine the UACR.

4. Biochemical analyses

Serum vitamin D, DKK-1 and hepcidin Measurement:

With the use of human ELISA kits provided by Eagle Biosciences, USA, Catalog No. VID31-K01; Biomedica, Austria, Catalog No. BI-20413; and DRG Diagnostics, Germany, Catalog No. EIA-5782, respectively, serum levels of vitamin D (25-hydroxyvitamin D3), DKK-1, and hepcidin were measured. According to the manufacturer's directions, each test was carried out.

Serum Oxidative Stress Markers Measurement:

Using commercially available colorimetric kits (Catalog Nos. GP 2524, SD 2521, MD 2529, and NO 2533, respectively) and following the manufacturer's instructions, the blood levels of GPx, SOD, LPER, and NO were determined. The kits were provided by Biodiagnostic, Giza, Egypt.

Serum glucose, HbA1c, Hb, lipid profile, UACR, creatinine and urea Measurement:

Enzymatic glucose kit, Catalog No. S1144A, provided by Spinreact, Spain, was used to measure the serum level of fasting blood glucose. An analytical kit with the catalogue number 30020430, provided by Agappy, India, was used to measure HbA1c. Hb was estimated by ABX pentra 60. Total cholesterol, triglyceride and HDL-C were determined by test kit Catalog No. 0168, 0141 and 0072, respectively, supplied by Human, Germany. The Friedewald formula was used to determine LDL-C: LDL-Cholesterol= Total cholesterol - (HDL Cholesterol + Triglyceride/5).

Using a protein test kit, albumin in urine was determined (Cromatest, Spain). Creatinine in urine was estimated by creatinine kit (Catalog No. CR 510, Randox, United Kingdom). Urinary albumin creatinine ratio (UACR): It is ratio of urinary albumin to urinary creatinine; it was expressed as milligram of albumin excreted per gram of urinary creatinine. The following equation was used to compute it: UACR (mg/g) = Albumin (mg/dl)/Creatinine (mg/dl) X 1000.

The concentrations of serum creatinine and urea were estimated using commercially available colorimetric kits (catalogue numbers CR 1251 and UR 2110, respectively), provided by Biodiagnostic, Giza, Egypt, and carried out in accordance with the manufacturer's guidelines.

Statistical Analysis

Categorical variables were presented by number and percent (n and %), whereas continuous variables were defined by mean and standard deviation (Mean, SD). Chi-squared test was used to examine the relationship between two qualitative variables. According to data distribution, comparison between groups was made using Student t test or Mann Whitney test. Correlation between variables was calculated using Pearson correlation coefficient. A two-tailed p value < 0.05 was considered statistically significant. All statistical analyses were completed using IBM SPSS software version 20.0 (SPSS Inc, Chicago, IL, USA) and GraphPad Prism 7 Software (San Diego, California, USA). ROC curve and cutoff value were calculated by MedCalc software version 14.

RESULTS

Demographic, clinical and biochemical data of study participants:

Table 1 provides an overview of the clinical and biochemical characteristics of the T2DM patients and controls in this study. When compared to controls,

T2DM patients had considerably greater levels of BMI, DKK1, hepcidin, LPER, NO, FBG, HbA1c, TC, TG, LDL-C, UACR, creatinine, and urea; but significantly lower levels of vitamin D, GPx, SOD, HDL-C, and Hb. Considering age and sex, there was no discernible difference between the two groups.

DN patients showed significantly greater age, duration of disease, DKK1, hepcidin, LPER, NO, FBG, HbA1c, TG, TC, LDL-C, UACR, creatinine and urea; while they showed significantly lower levels of vitamin D, GPx, SOD, Hb, and HDL-C, as compared to No DN patients.

Table (1): Demographic, clinical and biochemical characteristics of T2DM patients and controls

Variable	T2DM patients			Controls (n=30)	P1 Value	P2 Value
	All T2DM patients (n=55)	T2DM patients with DN (n=35)	T2DM patients without DN (n=20)			
Demographic and clinical characteristics of T2DM patients and controls						
Age (Years)	51.73±9.53	54.29 ±9.63	47.25 ±7.69	48.93 ±11.15	0.23	**<0.01**
Sex Male n (%) Female n (%)	 28 (50.9%) 27 (49.1%)	 17 (48.6 %) 18 (51.4 %)	 11 (55 %) 9 (45 %)	 18 (60 %) 12 (40 %)	 0.42	 0.65
Duration (Years)	6.77±2.28	7.64 ±2.13	5.16 ±1.56	-	-	**<0.001**
BMI (kg/m^2)	31.73 ±5.55	32.27 ±5.85	30.78 ±4.96	27.6 ±3.30	**0.001**	0.34
Biochemical characteristics of T2DM patients and controls						
Vitamin D (ng/ml)	10.1 ±2.3	7.79 ±1.91	14.13 ±3.42	43.21 ±10.34	**< 0.001**	**< 0.001**
DKK-1 (pmol/L)	30.73 ±7.1	34.43± 8.3	24.25± 5.25	7.35±1.32	**< 0.001**	**< 0.001**
Hepcidin (ng/ml)	15.02± 3.52	16.82± 3.10	11.87±2.84	8.04± 1.82	**< 0.001**	**< 0.001**
GPx (IU/ml)	107.76±21.92	99.77 ±20.76	121.74±16.46	165.4±27.25	**< 0.001**	**< 0.001**
SOD (IU/ml)	86.97±21.03	79.84±19.43	99.45±19.35	252.19±58.85	**< 0.001**	**0.001**
LPER (µmol/L)	9.15±2.19	10.35±2.45	7.05 ±1.24	4.63±1.09	**< 0.001**	**0.005**
NO (nmol/L)	13.15±3.21	14.19±3.18	11.32±2.61	5.34±1.2	**< 0.001**	**0.002**
FBG (mg/dl)	222.68± 47.48	259.15 ± 48.78	158.86± 20.88	93.97± 10.77	**< 0.001**	**< 0.001**
HbA1c (%)	9.13± 2.20	10.35± 2.21	7.01± 0.83	5.53± 1.14	**< 0.001**	**< 0.001**
Hb (g/dl)	10.23± 2.18	9.55± 1.72	11.41± 2.42	11.6± 1.66	**0.004**	**< 0.001**
TC (mg/dl)	229.89± 54.58	259.53±47.87	178.02± 39.25	149.38±17.47	**< 0.001**	**< 0.001**
TG (mg/dl)	261.32± 63.61	286.14± 66.69	217.880± 52.28	172.25±11.97	**< 0.001**	**< 0.001**
LDL-C (mg/dl)	169.13± 38.71	191.26 ±43.31	130.41 ±29.72	90.66± 9.98	**< 0.001**	**< 0.001**
HDL-C (mg/dl)	30.62± 7.24	27.03± 5.73	36.9± 5.0	45.17± 7.53	**< 0.001**	**< 0.001**
UACR (mg/g)	627.07±51.71	970.09±39.10	26.8±2.42	19.7±4.75	**<0.001**	**<0.001**
Creatinine (mg/dl)	2.19±0.43	2.97±0.53	0.84±0.14	0.96±0.21	**<0.001**	**<0.001**
Urea (mg/dl)	72.47±7.24	97.49±20.55	28.7±4.04	22.6±5.49	**<0.001**	**<0.001**

Data are represented as mean ± SD or numbers and percentages. P1: Comparison between entire T2DM patients and controls. P2: Comparison between T2DM patients with and without diabetic nephropathy.

Abbreviations: T2DM: Type 2 diabetes mellitus, BMI: Body mass index, DKK1: Dickkopf-1, GPx: Glutathione peroxidase, SOD: Superoxide dismutase, LPER: Lipid peroxide, NO: Nitric oxide, FBG: Fasting blood glucose, HbA1c: Glycated hemoglobin, Hb: Hemoglobin, TC: Total cholesterol, TG: Triglyceride, HDL-C: High density lipoprotein-cholesterol, LDL-C: Low density lipoprotein-cholesterol, UACR: Urine albumin creatinine ratio.

Serum levels of the studied biochemical parameters in the T2DM patients regarding duration of disease and degree of glycemic control

Comparison of studied biomarkers of T2DM patients as regards disease duration and glycemic control is displayed in table (2). T2DM patients having duration of disease more than 6 years showed significantly greater levels of DKK1, hepcidin, LPER and NO, FBG, HbA1c, TC, LDL-C, Creatinine, urea; while they displayed significantly lower levels of vitamin D, GPx, SOD, Hb and HDL-C compared to T2DM patients whose disease has been present for less than 6 years.

Concerning degree of glycemic control, patients with T2DM who have poor glycemic control exhibited increased levels of DKK1, hepcidin, NO, LDL-C, LPER, FBG, HbA1c, TC, creatinine and urea; however, they showed significantly lower levels of vitamin D, GPx, SOD and HDL-C as opposed to T2DM patients with good glycemic control.

Table (2): Comparison of biochemical characteristics of T2DM patients as regards duration of disease and glycemic control

	Duration of disease			**Glycemic control**		
	T2DM for less than 6 years (n=24)	**T2DM for more than 6 years (n=31)**	**P Value**	**Good glycemic control (HBA1c ≤7) (n=23)**	**Poor glycemic control (HBA1c >7) (n=32)**	**P Value**
Vitamin D (ng/ml)	13.15±3.28	7.74±1.79	**<0.001**	13.99± 3.26	7.3±1.35	**<0.001**
DKK-1 (pmol/L)	26.47±6.32	34.03±8.69	**<0.001**	26.02± 6.45	34.12± 8.84	**<0.001**
Hepcidin (ng/ml)	12.08±3.01	17.29±4.27	**<0.001**	12.72± 3.11	16.67± 4.01	**<0.001**
GPx (IU/ml)	114.98±18.78	101.952±22.91	**0.03**	123.48± 14.54	96.46± 19.28	**<0.001**
SOD (IU/ml)	93.45± 17.88	81.96± 19.85	**0.03**	100.88± 15.76	76.98± 18.55	**<0.001**
LPER (µmol/L)	7.23± 1.32	10.64±2.36	**<0.001**	6.24±1.21	11.24±2.74	**<0.001**
NO (nmol/L)	11.92± 2.52	14.09± 3.13	**<0.01**	11.72± 2.31	14.17± 2.96	**0.002**
FBG (mg/dl)	179.51± 40.38	256.094± 62.59	**<0.001**	174.09± 43.92	257.6± 60.62	**<0.001**
HbA1c (%)	7.51± 1.77	10.39± 2.13	**<0.001**	6.7± 0.37	10.88± 1.64	**<0.001**
Hb (g/dl)	11.38± 2.36	9.34± 1.54	**0.001**	11.51± 2.43	9.31± 1.4	**<0.001**
TC (mg/dl)	203.87± 48.32	250.03± 53.12	**0.002**	195.52± 46.83	254.59± 51.94	**<0.001**
TG (mg/dl	231.51± 55.38	284.4± 68.24	**0.003**	233.03± 54.65	281.66± 58.83	**0.003**
LDL-C (mg/dl)	150.27± 35.14	183.74± 44.2	**0.047**	143.56± 33.57	187.52± 45.74	**<0.001**
HDL-C (mg/dl)	34.2± 7.24	27.84±6.0	**<0.001**	34.69± 6.37	27.69±6.44	**<0.001**
Creatinine (mg/dl)	1.53±0.34	2.71±0.61	**0.001**	1.41±0.31	2.76±0.62	**<0.001**
Urea (mg/dl)	52.33±12.05	88.06±20.61	**<0.001**	46.69±10.41	91.0±21.61	**<0.001**

Values are mean±SD. T2DM:-Type 2 diabetes mellitus, DKK1: Dickkopf-1, FBG: Fasting blood glucose, TC: Total cholesterol, TG: Triglyceride, HDL: High density lipoprotein-cholesterol, LDL: Low density lipoprotein-cholesterol, GPx: Glutathione peroxidase, SOD: Superoxide dismutase, LPER: Lipid peroxide, NO: Nitric oxide,

The diagnostic efficacy of studied biomarkers

To evaluate the examined biomarkers' diagnostic efficiency for T2DM discrimination, the ROC curve was conducted (Table 3). Interestingly, vitamin D, DKK1and SOD showed the highest predictive value for T2DM development, as they obtained the highest AUC.

The ability of the investigated factors to identify DN from No DN patients was also assessed using the ROC curve as shown in table (3). Vitamin D and hepcidin showed the highest predictive value for DN development.

Table (3): Diagnostic performance of the studied parameters for distinguishing patients

	Sensitivity %	Specificity %	Cutoff	AUC	PPV %	NPV %	Accuracy %
Discriminating T2DM from Controls							
Vitamin D (ng/ml)	94.5	96.7	<20	0.99	98.1	90.6	91.2
DKK1(pmol/L)	98.2	93.3	>9.1	0.99	96.4	96.6	91.5
Hepcidin (ng/ml)	78.2	93.3	>10.5	0.89	95.6	70.0	71.5
GPx (IU/ml)	80	93.3	<130.5	0.95	95.7	71.8	73.3
SOD (IU/ml)	98.2	93.3	< 132.2	0.99	96.4	96.6	91.5
LPER (µmol/L)	72.7	93.3	>6.1	0.84	95.2	65.1	66.1
NO (nmol/L)	96.4	93.3	>6.7	0.90	96.4	93.3	89.7
Discriminating DN from No DN							
	Sensitivity %	**Specificity %**	**Cutoff**	**AUC**	**PPV %**	**NPV %**	**Accuracy %**
Vitamin D (ng/ml)	91.4	70	<10.2	0.88	84.2	82.4	61.4
DKK1(pmol/L)	60	75	>28.7	0.72	80.8	51.7	35
Hepcidin (ng/ml)	80	95	>15.8	0.83	96.6	73.1	75
GPx (IU/ml)	57.1	95	<96.8	0.80	95.2	55.9	52.1
SOD (IU/ml)	57.1	95	<76.4	0.77	95.2	55.9	52.1
LPER (µmol/L)	57.1	90	>9.5	0.73	90.9	54.5	47.1
NO (nmol/L)	68.6	75	>13.3	0.75	82.8	57.7	43.6

Logistic regression analysis: risk factors for DN in T2DM patients

In the univariate logistic regression analysis, DN was used as the dependent variable and vitamin D, DKK1, hepcidin, GPx, SOD, LPER, and NO were used as independent variables and found to be all risk factors for DN. In multivariate logistic analysis, only hepcidin was significantly associated with DN (Table 4). It was found to be the independent predictor of DN in T2DM patients and about 56.8% of the variation in the total impact score was explained by these explanatory variables (R Square= 0.568).

Table (4): Univariate and multivariate regression analysis to determine the independent predictors of diabetic nephropathy in T2DM patients

	Univariate analysis			Multivariate analysis		
Characteristics	**OR**	**95% CI**	**P Value**	**OR**	**95% CI**	**P Value**
Vitamin D (ng/ml)	0.525	0.356- 0.773	**0.001**	0.263	0.063-1.091	0.07
DKK1(pmol/L)	1.092	1.020- 1.170	**0.01**	1.076	0.944-1.227	0.27
Hepcidin (ng/ml)	1.297	1.115- 1.508	**0.001**	1.526	1.059-2.198	**0.02**
GPx (IU/ml)	0.946	0.915- .978	**0.001**	0.897	0.719-1.119	0.33
SOD (IU/ml)	0.956	0.927- 0.985	**0.003**	1.116	0.951-1.310	0.18
LPER (µmol/L)	1.251	1.061- 1.476	**0.008**	0.713	0.389-1.306	0.27
NO (nmol/L)	1.340	1.087- 1.652	**0.006**	1.215	0.826-1.790	0.32

Correlations between studied parameters

Our research revealed a significant positive correlation between vitamin D and GPx and SOD (Figure 1 C, D). However, Significant negative correlation was detected between vitamin D and DKK1; hepcidin; LPER, NO, HbA1c and UACR (Figure 1 A, B, E, F, G, H).

Significant positive correlation was detected between DKK1 and Hepcidin; HbA1c; and UACR (Figure 2 A, D, E respectively); Also, a significant positive correlation was found between serum hepcidin level and NO; HbA1c; and UACR (Figure 2 G, H, I). On the other hand, significant negative correlation was found between serum DKK1 level and GPx; and SOD (Figure 2 B C,). Also, significant negative correlation was found between serum hepcidin level and SOD (Figure 2 F).

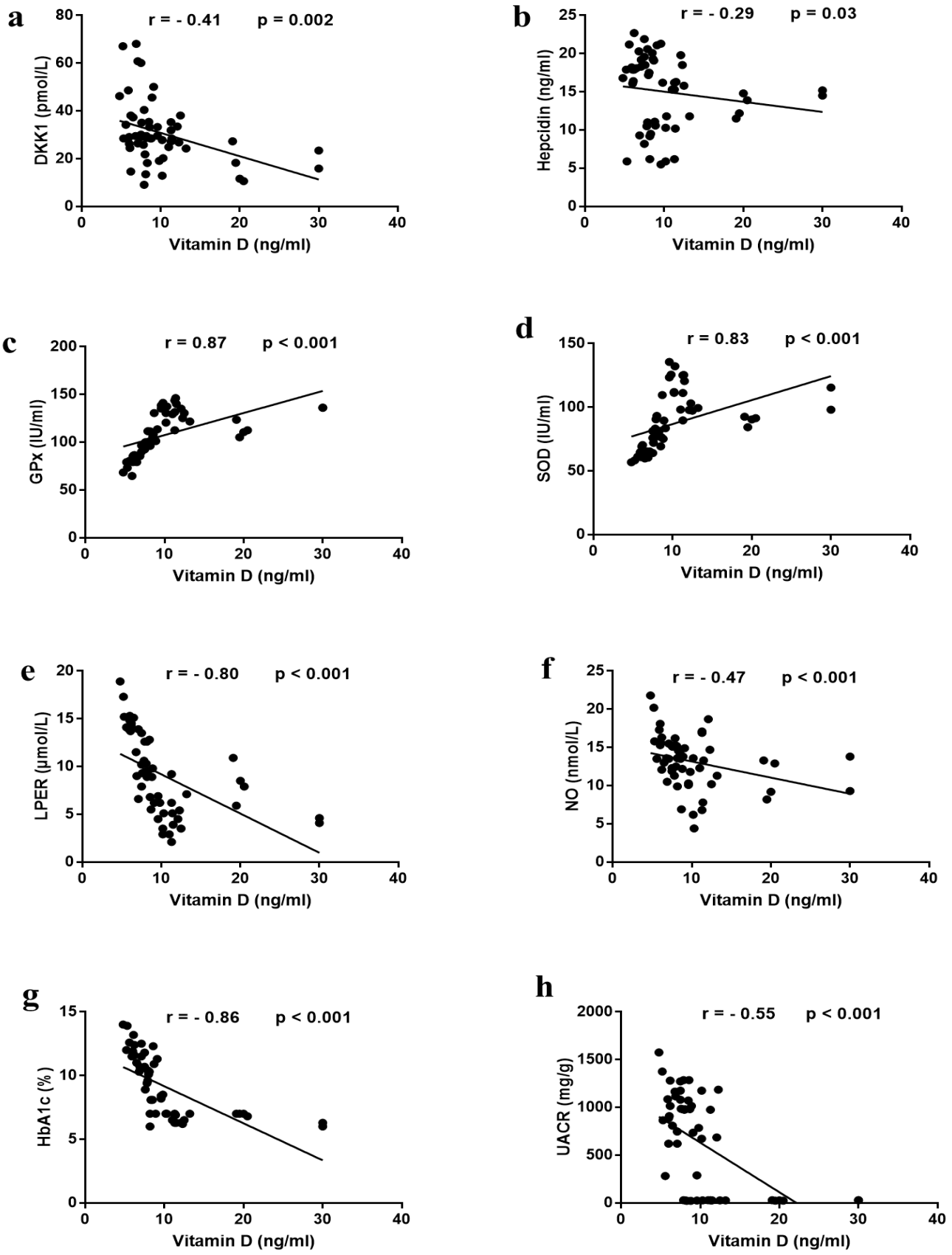

Figure (1): Correlation between: A) Serum vitamin D level and DKK1; B) Serum vitamin D level and hepcidin; C) Serum vitamin D level and GPx; D) Serum vitamin D level and SOD; E) Serum vitamin D level and LPER; F) Serum vitamin D level and NO; G) Serum vitamin D level and HbA1c; H) Serum vitamin D level and UACR among 55 T2DM patients

r: Spearman correlation factor.

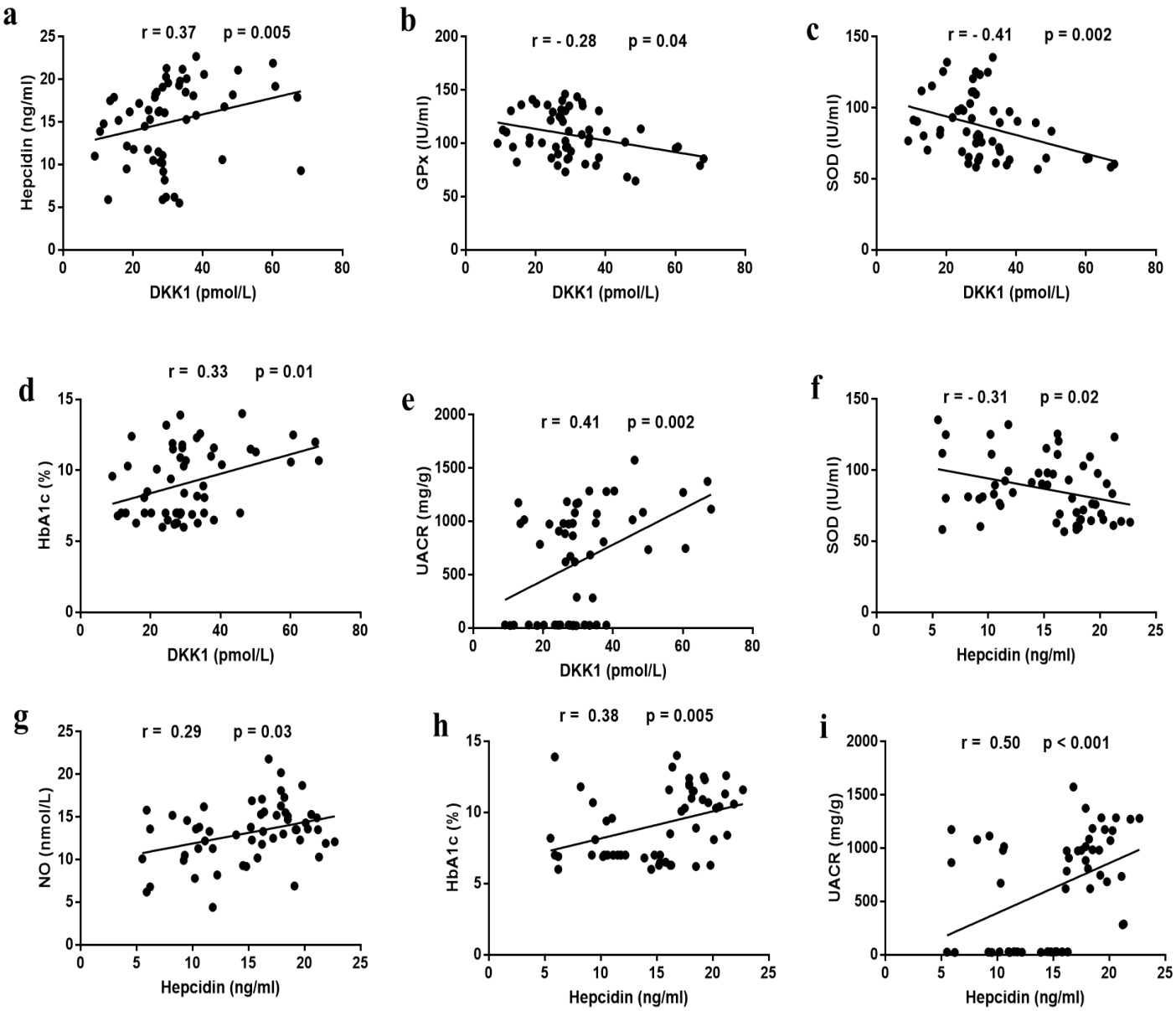

Figure (2): Correlation between A) serum DKK1 level and hepcidin; B) serum DKK1 level and GPx; C) serum DKK1 level and SOD; D) serum DKK1 level and HbA1c; E) serum DKK1 level and UACR; F) Serum hepcidin level and SOD; G) Serum hepcidin level and NO; H) Serum hepcidin level HbA1c; I) Serum hepcidin level and UACR

r: Spearman correlation factor.

DISCUSSION

One of the most prominent microvascular consequences of DM is DN. Development of novel assays for the diagnosis of DN has always been a top goal in the research on DM complications in order to avoid the bad outcome. Our bodies use vitamin D for a wide range of functions, including immune system function, bone remodeling, and regulation of mineral equilibrium. Aside from these functions, significant direct and indirect effects of vitamin D on β -cell functional role, insulin sensitivity, and secretion have been observed [(12)].

The results of the current study proved that serum level of vitamin D was significantly lower in T2DM patients as compared to controls and also in DN patients as compared to No DN patients. Vitamin D demonstrated diagnostic accuracy in the differentiation of T2DM and DN. Our findings agreed with those of **Zhao *et al.*, [(13)].** According to **Morró *et al.*[(14)],** a deficiency of vitamin D has been linked to T2DM. It also contributes to a number of DM problems, including DN.

The present study revealed that vitamin D levels were significantly decreased in T2DM patients with a disease duration of more than 6 years and those with poor glycemic control. In parallel, **Salih *et al.*** [(15)] observed lower levels of vitamin D in T2DM patients who had long disease duration and had poor glycemic

control. Also, **Wu *et al.*** [16] illustrated that inadequate vitamin D levels have been linked to poor glycemic control in T2DM and that vitamin D treatment may help to improve glycemic control. However, the study conducted by **Oraby *et al.*** [17] did not reveal difference in the vitamin D level between T2DM patients as regards duration and glycemic control.

We discovered in this research that vitamin D has positive correlation with GPx and SOD and negative correlation with LPER, NO and HbA1c. Vitamin D can play an antioxidant role by preventing the production of free radicals [18]. In agreement with our results, **Alatawi *et al.*** [19] elucidated that diabetic rats showed a significant decrease in SOD and GPx but a significant elevation in MDA, the situation was reversed after treatment of diabetic rats with vitamin D supplementation indicating that vitamin D played a crucial role in the protection of tissues from damage by free radicals. Furthermore, we noticed inverse relationship between vitamin D and UACR, a finding supported by **Liang *et al.*** [20]**.**

There are two types of Wnt signalling pathways: β-catenin-dependent (canonical) and β-catenin-independent (non-canonical). The formation of a complex on the cell membrane between Wnts, their cognate receptor Frizzled (Fzd), and coreceptor low-density lipoprotein receptor-related protein 5/6 (LRP5/6) triggers the activation of canonical Wnt signalling changing a number of biological processes [21]. Wnt signalling plays a significant role in regulating insulin sensitivity, and its dysregulation has been related to progression of diabetes [22].

We discovered that DKK1 levels in T2DM patients were considerably higher in comparison to controls. DKK1 levels were also greater in DN patients than in non-DN patients. DKK1 displayed great diagnostic performance for T2DM and DN. These findings indicated the possible role of DKK1 in diagnosis of T2DM and suggest that serum DKK1 may be a predictor of the presence of DN in this population. These findings were consistent with those of **Lattanzio *et al.*** [23] who noticed increased levels of circulating DKK1 in T2DM patients. The complex that arises when DKK-1 connects to Kremen-2 prevents Wnt signaling. High glucose levels cause DKK-1 and Kremen-2 to be synthesized, which results in DN. As a consequence, blocking the DKK-1/Wnt signalling pathway could be a promising novel therapeutic method to slow the course of mesangial cellular damage in diabetic glomerular disease [7].

DKK1 serum levels were considerably higher in T2DM patients with more than 6 years of disease duration and with poor glycemic control. In accordance with our findings, **Faienza *et al.*** [24] demonstrated that serum DKK-1 levels were substantially associated to HbA1c% and diabetes duration in infants and adolescents with T1DM and established the involvement of DKK-1 in the altered glycemic control. On the other hand, **Lattanzio *et al.*** [23] found no differences in the plasma DKK-1 concentration between individuals with newly diagnosed DM and those with long-standing DM, as well as between those with good and poor glycemic control. The discrepancies in the patient's characteristics may be to blame for this disparity between findings. T2DM treatment that targets Wnt antagonism might be an alternative, according to an experimental study by **Li *et al.*** [25] that showed suppression of the Wnt antagonist DKK-2 lowers baseline concentrations of blood glucose and enhance tolerance of glucose. Therefore, more research into DKK-1 targeting in T2DM is warranted.

Significant positive correlation was found between DKK1 and UACR. This was consistent with study done by **Lin *et al.*** [26] demonstrated that serum DKK1 levels were higher in diabetic rats. DKK1 was strongly expressed in podocytes, tubular cells, and glomerular mesangial cells in diabetic kidneys, demonstrating that DKK1 is expressed by different cell types in the renal tissue. Diabetes-related kidney damage, deposition of fibrotic matrix in in kidney, and protein excretion in the urine are all reversed when DKK1 expression is reduced in vivo. High glucose concentrations promoted renal tissue degeneration, which was reduced by interrupting DKK1 function.

Our data revealed a substantial inverse relationship between serum DKK1 levels and GPx and SOD. Protein byproducts created during oxidative stress encourage the production of ROS, which triggers the motivation of β-catenin and the induction of Wnt ligands. As a result, podocytes become damaged and dysfunctional, impairing glomerular filtration and initiating proteinuria. Wnt/β-catenin is a critical mediator of proteinuria and dysfunction of podocytes brought on by oxidative stress [27].

Iron metabolism and diabetes are influenced by one another, according to research. In a hyperglycemic condition, several of the bodily processes in charge of maintaining iron homeostasis are disturbed. On the other side, too much iron affects the release and function of insulin. Having too much iron in your system encourages the emergence of T2DM and glucose intolerance [28].

The current study's findings demonstrated an increase in hepcidin levels in T2DM patients compared to controls with higher levels of hepcidin in DN patients than in No DN patients. Moreover, Hb levels declined considerably in patients with T2DM as compared to controls and in DN patients than in No DN patients. Hepcidin levels were also observed to be considerably higher with increased duration of T2DM and poor glycemic control. These findings were in matching with previous investigators [28]. Also another research by **Pappa *et al.*** [29] reported that anaemia in people with

DKD develops earlier and is more severe than in people without the disease.

By Fenton and Haber-Weiss chemistry, an increase in the amount of free ferrous iron results in the production of ROS. The most reactive oxygen radical is the hydroxyl radical (OH), which is created by the Fenton process. It may easily combine with nearby biological molecules to cause considerable harm [(30)]. OH is a significant species that damages DNA, proteins, and lipids in cell membranes and damages tissue, resulting in insulin resistance and ultimately β-cell failure [(31)]. This was corroborated by our research that revealed a substantial increase in OS in T2DM patients. Also, hepcidin correlated significantly positively with NO and significantly negatively with SOD.

Additionally, observational and epidemiologic studies have already identified the link between vitamin D insufficiency and anaemia. Vitamin D suppresses hepcidin mRNA production in monocytes and hepatocytes by directly interacting with vitamin D response elements on the hepcidin gene promotor. By inhibiting proinflammatory cytokines, which promote the synthesis of hepcidin, vitamin D also indirectly affects hepcidin expression [(32)]. This was reinforced by our research, which found a substantial inverse relationship between hepcidin and vitamin D.

Increased OS is thought to characterize diabetes. OS is regarded to be one of the fundamental causes of diabetic microvascular and macrovascular complications [(33)]. There are a number of hypothesized pathways connecting hyperglycemia with increased ROS production. These mechanisms include rise in superoxide anion radical generation (O_2^-) in the mitochondria, activation of the nuclear factor kappa B (NF-κB) signaling pathway that causes inflammation and increases the production of ROS in phagocytes, an increase in the flow of glucose via the polyol pathway, as well as the production of advanced glycation end products (AGE), which increases OS [(34)].

In the present study, by determining the antioxidant enzymes, LPER and NO, we have addressed the problem of increased OS in T2DM patients. When compared to controls, patients with T2DM had significantly higher mean levels of LPER and NO and significantly lower mean levels of GPx and SOD. DN patients showed significantly increased levels of LPER and NO and a significant decrease in the mean levels of GPx and SOD, as compared to No DN patients. Additionally, T2DM patients of a duration exceeding 6 years and poor glycemic control showed increased levels of LPER and NO and decreased levels of GPx and SOD in comparison with T2DM patients who have had the disease for less than 6 years and good glycemic control. These findings were in line with previous studies [(35)]. These results strengthen the potential for OS to play a role in T2DM.

CONCLUSION

In T2DM patients, particularly those with DN, long duration, and poor glycemic control, DKK1, hepcidin, LPER, and NO were raised whereas vitamin D, GPx, and SOD were depressed. These biomarkers may be useful in the diagnosis and prediction of serious disorders such T2DM and DN. They may also have therapeutic promise in the future.

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