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Original Article

Urinary 20-HETE: A prospective Non-Invasive prognostic and diagnostic marker for diabetic kidney disease

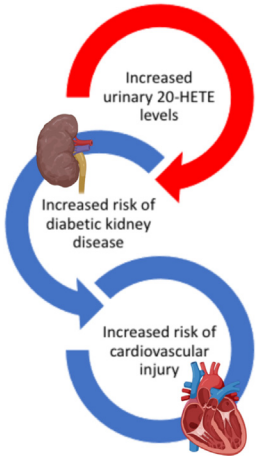
Pamela Houeiss [a,b,1], Rachel Njeim [a,b,1], Hani Tamim [c], Ahmed F. Hamdy [d], Tanya S. Azar [a,b], William S. Azar [a,b,e], Mohamed Noureldein [a,b], Youssef H. Zeidan [f], Awad Rashid [d], Sami T. Azar [b,c], Assaad A. Eid [a,b,*]

[a] Department of Anatomy, Cell Biology and Physiological Sciences, Faculty of Medicine, American University of Beirut, Lebanon
[b] AUB Diabetes Program, Faculty of Medicine, American University of Beirut, Lebanon
[c] Department of Internal Medicine, Faculty of Medicine, American University of Beirut, Lebanon
[d] Department of Nephrology, Hamad Medical Corporation, Doha, Qatar
[e] Department of Physiology and Biophysics, Georgetown University School of Medicine, Washington, DC, USA
[f] Department of Radiation Oncology, Faculty of Medicine, American University of Beirut, Lebanon

HIGHLIGHTS

- Identifying urinary 20-HETE as a prospective diagnostic/prognostic test for diabetes and its complications
- Urinary 20-HETE/Cr ratio positively correlates with diabetes mellitus
- Urinary 20-HETE/Cr ratio positively correlates with micro/macrovascular complications
- Urinary 20-HETE alteration is associated with diabetes-induced cardiovascular injury
- Urinary 20-HETE/Cr ratio correlates with the severity of kidney injury

GRAPHICAL ABSTRACT



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ABSTRACT

Introduction: The identification and validation of a non-invasive prognostic marker for early detection of diabetic kidney disease (DKD) can lead to substantial improvement in therapeutic decision-making.
Objectives: The main objective of this study is to assess the potential role of the arachidonic acid (AA) metabolite 20-hydroxyeicosatetraenoic (20-HETE) in predicting the incidence and progression of DKD.
Methods: Healthy patients and patients with diabetes were recruited from the Hamad General Hospital in Qatar, and urinary 20-HETE levels were measured. Data analysis was done using the Statistical Package for Social Sciences (SPSS).
Results: Our results show that urinary 20-HETE-to-creatinine (20-HETE/Cr) ratios were significantly elevated in patients with DKD when compared to patients with diabetes who did not exhibit clinical signs of

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* Corresponding author at: American University of Beirut, Department of Anatomy, Cell Biology and Physiological Sciences, Faculty of Medicine and Medical Center, Bliss Street, 11-0236, Riad El- Solh 1107-2020, Lebanon.
E-mail address: ae49@aub.edu.lb (A.A. Eid).
[1] **The authors equally contributed to this work**.

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Microvascular Complications
Macrovascular Complications
20-HETE

kidney injury ($p < 0.001$). This correlation was preserved in the multivariate linear regression accounting for age, diabetes, family history of kidney disease, hypertension, dyslipidemia, stroke and metabolic syndrome. Urinary 20-HETE/Cr ratios were also positively correlated with the severity of kidney injury as indicated by albuminuria levels ($p < 0.001$). A urinary 20-HETE/Cr ratio of 4.6 pmol/mg discriminated between the presence and absence of kidney disease with a sensitivity of 82.2 % and a specificity of 67.1%. More importantly, a 10-unit increase in urinary 20-HETE/Cr ratio was tied to a 10-fold increase in the risk of developing DKD, suggesting a 20-HETE prognostic efficiency.
Conclusion: Taken together, our results suggest that urinary 20-HETE levels can potentially be used as non-invasive diagnostic and prognostic markers for DKD.

Introduction

Diabetic kidney disease (DKD) is one of the most serious complications of diabetes worldwide, affecting up to 40% of patients with diabetes. **The epidemiology of DKD varies widely** [1]**. The prevalence of DKD is on the rise particularly in low and middle-income countries. It is also increasing among children and adolescents paralleled with an increase in the prevalence of early-onset type 2 diabetes mellitus (T2DM). Early-onset T2DM is associated with faster progression to kidney disease due to possible genetic predisposition, coexisting risk factors, and longer exposure to hyperglycemia** [2]**. Genetic factors and gender differences may also affect the prevalence and the progression of DKD** [3]**. The burden of DKD is particularly elevated in the eastern Mediterranean region (EMR). In the Global Burden of Disease 2015 study, Tunisia, Saudi Arabia and Afghanistan had the highest disability-adjusted life years (DALY) for chronic kidney disease (CKD) due to DM in males and females. In the last 25 years, the mortality rate of DKD increased by 179% in the EMR** [4]**.**

DKD is the leading cause of end-stage renal disease and cardiovascular morbidity and mortality worldwide. Increased urinary albumin excretion, also known as albuminuria, is the earliest clinical manifestation of DKD and one of the most important prognostic risk factors for the progression of kidney disease [5]. Nonetheless, albuminuria lacks the necessary sensitivity and specificity to accurately predict the development and progression of DKD in patients with diabetes, which creates an essential need to develop alternative diagnostic markers with higher sensitivity and specificity [6]. We discuss herein a potential candidate: the arachidonic acid (AA) metabolite 20-hydroxyeicosatetraenoic (20-HETE).

The eicosanoid 20-HETE originates from AA by the action of cytochromes P450 (CYPs) 4A and 4F [7], which are regulated by hormones and epigenetic factors [8]. CYPs are predominantly expressed in the liver but are also present in the heart, lungs, gastrointestinal tract, vascular system and kidneys. In the kidneys, CYPs play a crucial role in homeostasis, in the regulation of vascular tone, and in the production of reactive oxygen species (ROS) [9-12]. 20-HETE, CYPs' biologically active metabolite, may possess a pro-hypertensive action, an anti-hypertensive action or a pro-oxidant action, depending on both the site of production (interlobular, cortical or preglomerular microvessels) and the site of action (vascular, glomerular or tubular) [9-12].

20-HETE undergoes glucuronidation in the liver prior to urinary excretion [8]. Levels of 20-HETE can be measured both in serum and in urine and are influenced by several factors that regulate 20-HETE synthesis, metabolism and elimination [12,13].

20-HETE plays a key role in maintaining homeostasis and in regulating renal, pulmonary, cardiac and vascular functions [14]. Dysregulation of 20-HETE is thought to be involved in the pathophysiology of many diseases including hypertension, CKD and DKD [12,15,16]. Very few studies have reported a correlation between serum 20-HETE and hypertension or CKD [17]. The role of 20-HETE, specifically that of urinary 20-HETE, as a prognostic and/or diagnostic marker for kidney disease in general and for DKD in particular remains poorly investigated.

To our knowledge, our study is the first to show that alteration in urinary 20-HETE-to-creatinine (20-HETE/Cr) ratio correlates with DKD and is associated with other diabetic complications such as diabetic retinopathy. Our study also finds that urinary 20-HETE/Cr ratio is associated with diabetes-induced cardiovascular injury. Taken together, our data suggest that 20-HETE may serve as a potential predictor and diagnostic tool for diabetes-induced kidney injury and other micro- and macrovascular diabetic complications.

Materials and Methods

Study Design

In this cross-sectional study, patients were recruited from **the outpatient nephrology clinics** at Hamad General Hospital in Qatar after Institutional Review Board approval (study no. 11313/11). 20-HETE measurements were conducted at the American University of Beirut, Beirut, Lebanon **(**Fig. 1**)**.

Study subjects

Subjects were considered eligible for the study if they were between 18 and 74 years of age and were able to provide an informed consent **(**Fig. 1**)**. No restrictions on either race or sex were included in this study. The exclusion criteria covered substance abuse, lactation, pregnancy or participation in an interventional trial.

Ethics statement

All experiments were conducted according to the ethical policies and procedures approved by the Institutional Review Board (study no. 11313/11) at Hamad General Hospital in Qatar. Informed consent was obtained from the patients.

Data collection

Data on socio-demographics, past medical history and family history were collected from medical records. T2DM was diagnosed based on the American Diabetes Association (ADA) guidelines (HbA1c $\geq$ 6.5%, fasting glucose levels $\geq$ 126 mg/dl and/or random glucose level $\geq$ 200 mg/dl) [18]. Kidney injury in patients with diabetes was defined according to the gold standard of diagnosis as presenting with albuminuria (albumin-to-creatinine ratio (ACR) above 3 mg/mmol) and/or an abnormal estimated glomerular filtration rate (eGFR) [19]. ACRs were classified into 3 categories based on severity using 3 mg/mmol and 30 mg/mmol as cutoffs**. Severity of kidney injury was classified by stages based on GFR as per the**

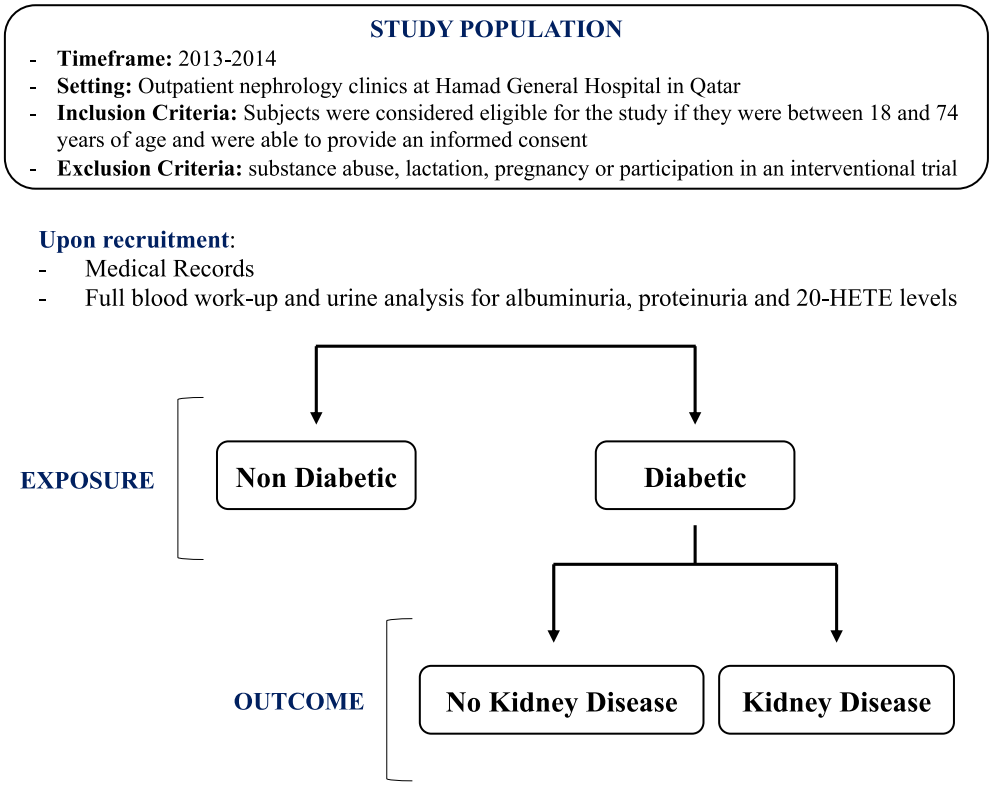

Fig. 1. Schematic of Study Design.

National Kidney Foundation recommendations [20]. Metabolic syndrome was defined according to the criteria suggested by the American Heart Association and the National Heart Lung and Blood Institute [21]. Urine samples for laboratory testing were collected at the time of the clinic visit. All data were coded using a unique identifier that kept the subject's identity anonymous.

Assessment of urinary 20-HETE levels

Urinary 20-HETE levels were measured using the 20-HETE Glucuronide ELISA kit (Detroit R&D, Inc., USA) according to the manufacturer's protocol [22]. Validation experiments on 20-HETE urinary concentrations by gas chromatography-mass spectrometry (GC–MS) using electron capture negative chemical ionization, expressed in pmol/l, yielded similar values. Levels were done in triplicates, and the mean of the values was used to avoid any measurement bias. Urinary 20-HETE levels were standardized by dividing 20-HETE urinary levels (pmol/l) by urine creatinine concentration (mg/L). All analytical measurements were performed by a technician who was blinded to the study purpose.

Statistical analysis

Data analysis was done using the Statistical Package for Social Sciences (SPSS) version 25 (IBM, USA). Baseline characteristics of the study population were determined using descriptive statistics. Results of descriptive statistics were reported using mean ± standard deviation (SD) for the continuous variables and frequency or percent for the categorical values. The association between urinary 20-HETE/Cr ratios and co-variates was evaluated using either independent *t*-test, one-way ANOVA or correlation. The second part of the analysis was a multivariate regression analysis **adjusted for possible confounding variables**. Multiple linear regression analysis with backward analysis was used to determine whether clinical variables had an independent effect on urinary 20-HETE/Cr ratios. Models were constructed using the variables that were significantly associated with urinary 20-HETE/Cr ratios in the univariate analysis. A model was done using all subjects. Models were considered significant if the *p* value was below 0.05. Beta estimates, confidence intervals, and *p* values were reported for the retained variables. Adjusted R-squared was reported for each model to describe the extent to which each model determines or explains the urinary 20-HETE/Cr ratios. Another model was done using the logistic regression with Backward LR analysis to determine the independent variables that predict the development of kidney disease in patients with diabetes. Variables that were significantly associated with DKD were included in the analysis. Odds Ratio, confidence interval and *p* value were reported for the retained variables. ROC curve analysis was used to quantify 20-HETE performance, both graphically and statistically. The Area Under the Curve (AUC) was calculated to determine whether urinary 20-HETE/Cr ratios discriminate between the presence and absence of kidney injury in diabetes. The optimal cutoff value for 20-HETE was chosen using the J statistic Youden index. The ROC curve was also used to determine the capacity of the DKD model to diagnose kidney disease using the predicted probabilities for each patient.

Results

Urinary 20-HETE level and its association with gender, age, and ethnicity

Out of the 182 patients recruited in this study, 54.9% were males and 45.1% were females. Patients came from different countries in Asia and Africa with the majority from the Middle East and North Africa (MENA) region (62.1%). The other recruited patients came from Nepal, the Philippines, Sri Lanka, Pakistan, India and Bangladesh. Baseline characteristics of the subjects are presented in Table 1. Analyzing the mean variations of urinary 20-HETE/Cr ratios with sociodemographic characteristics revealed a significant

Table 1
Association of urinary 20-HETE/Cr ratio with various patient characteristics.

		All patients	Urinary 20-HETE/Cr ratioMean (pmol/mg) ± SD or R correlation factor	p value
Socio-demographics				
Gender (n = 182)	Male	100 (54.9%)	17.2 ± 23.6	0.428
	Female	82 (45.1%)	14.6 ± 18.9	
Ethnicity (n = 182)	Southeast Asia	10 (5.5%)	7.5 ± 7.4	0.525
	Middle East	97 (53.3%)	15.8 ± 21.4	
	South Asia	52 (28.6%)	17.5 ± 23.3	
	Africa	23 (12.6%)	17.3 ± 22.9	
Race (n = 182)	Arab	113 (62.1%)	15.8 ± 21.1	0.579
	Indian	52 (28.6%)	17.7 ± 23.3	
	Chinese	2 (1.1%)	7.1 ± 2.5	
	Filipino	10 (5.5%)	7.5 ± 7.4	
	Persian	5(2.7%)	35.7 ± 15.9	
Age (years) (n = 182) (R correlation)		47.3 ± 11.4	0.325	**<0.001**
Comorbidities				
Diabetes (n = 182)	Yes	128 (70.3%)	21.9 ± 23.3	**<0.001**
Diabetes duration (years) (n = 126) (R correlation)		10.5 ± 9.2	0.519	**<0.001**
HbA1c Level (mmol/mol) (n = 124)	<53	49 (60.5%)	25.4 ± 24.2	**0.056**
	>53	75(39.5%)	17.1 ± 21.8	
BMI category (n = 182)	underweight	1 (0.5%)	19.1 ± 0	0.901
	normal weight	42 (23.1%)	18.1 ± 23.7	
	overweight	57 (31.3%)	15.8 ± 22.9	
	obese	82 (45.1%)	15.1 ± 19.7	
Family history of diabetes (n = 182)	No family history	76 (41.8%)	11.1 ± 21.2	**0.016**
	first degree	104 (57.1%)	19.2 ± 21.2	
	second degree	2 (1.1%)	37.1 ± 25.4	
Kidney disease in Patients with diabetes (n = 99)	Yes	78 (75.7%)	27.7 ± 26.4	**<0.001**
Family history of kidney disease (n = 182)	yes	31 (17%)	31.8 ± 18.4	**<0.001**
Diabetic retinopathy (n = 128)	yes	22 (17.2%)	33.8 ± 24.9	**0.008**
Diabetic neuropathy (n = 128)	yes	17 (13.3%)	30.3 ± 18.9	**0.114**
Hypertension (n = 182)	yes	79 (43.4%)	21.7 ± 24.4	**0.003**
Duration of hypertension (years) (n = 79) (R correlation)		6.8 ± 5.4	0.251	0.131
Coronary artery disease (n = 182)	yes	21 (11.5%)	30.1 ± 27.7	**0.019**
Peripheral vascular disease (n = 182)	yes	6 (3.3%)	30.1 ± 32.2	0.106
Stroke (n = 182)	yes	5 (2.7%)	42.8 ± 35.9	**0.005**
Dyslipidemia (n = 182)	yes	71 (39%)	23.4 ± 22.8	**<0.001**
Metabolic syndrome (n = 182)	Yes	120 (65.9)	20.8 ± 22.9	**<0.001**
Kidney disease (n = 148)	Absent	70 (47.3%)	4.8 ± 6.8	**<0.001**
	Diabetic kidney disease	75 (50.7%)	27.4 ± 26.7	
	Other causes	3 (2%)	1.8 ± 1.26	
Smoking duration (years) (R correlation)	25	8.8 ± 9.5	R = 0.314	0.126
Medications				
Oral hypoglycemic (n = 128)	yes	98 (76.6%)	19.1 ± 22.4	0.036
Insulin (n = 128)	yes	37 (28.9%)	30.4 ± 20	**<0.001**
Calcium channel blocker (n = 182)	yes	29 (15.9%)	20.1 ± 18.1	**0.273**
Beta blocker (n = 182)	yes	35 (19.2%)	25.6 ± 26.9	0.018
Diuretic HCT (n = 182)	yes	14 (7.7%)	21.4 ± 21.8	**0.333**
Drugs acting on RAS (n = 182)	ACEI or ARB	67 (36.8%)	20.2 ± 20.2	**0.049**
Antiplatelet: Aspirin (n = 182)	yes	48 (26.4%)	29.9 ± 28.4	**<0.001**
Statin (n = 182)	yes	75 (41.2%)	121.4 ± 21.7	**0.004**
Physical exam				
Systolic blood pressure (mmHg)	182	133.65 ± 18.34	0.224	**0.002**
Diastolic blood pressure (mmHg)	182	77.25 ± 9.7	−0.006	0.182

20-HETE, 20-hydroxyeicosatetraenoic acid; ACEI, angiotensin converting enzyme inhibitor; ARB, angiotensin II receptor blockers; BMI, body mass index; HCT, hydrochlorothiazide; RAS, renin-angiotensin system. p < 0.05 is statistically significant (in bold font).

difference in urinary 20-HETE/Cr ratio between different age categories; levels were higher among those between 45 and 74 years compared to those between 21 and 44 years, with mean values of urinary 20-HETE/Cr ratio increasing from 7.8 to 18.6, reaching 52.5 pmol/mg in the elderly ($p < 0.001$). The variations of 20-HETE levels in urine were not significantly affected by region ($p = 0.525$), gender ($p = 0.428$), or race ($p = 0.579$) (Table 1).

Urinary 20-HETE levels positively correlate with diabetes and its co-morbidities

A major finding in our study is the correlation of 20-HETE with diabetes and diabetes duration. Our data reveal that urinary 20-HETE/Cr ratio is significantly higher in patients with diabetes as compared to patients without diabetes (21.9 pmol/mg *vs* 2.1 pmol/mg; $p < 0.001$). Urinary 20-HETE/Cr ratio positively correlates with diabetes duration with a correlation factor of 0.519 ($p < 0.001$) (Table 1). These observations were independent from patient obesity status as urinary 20-HETE/Cr ratios did not correlate with the subjects' body mass index (BMI) ($p = 0.794$).

Of interest, when considering microvascular complications, patients suffering from retinopathy and nephropathy had higher urinary 20-HETE/Cr ratios with a mean difference of 33.8 and 27.7 pmol/mg, respectively. Likewise, other co-morbidities were associated with an increase in urinary 20-HETE/Cr ratio. For instance, urinary 20-HETE/Cr ratio in hypertensive patients was

twice as elevated as in non-hypertensive subjects (p = 0.003), and patients with coronary artery disease had a urinary 20-HETE/Cr ratio of 30.1 pmol/mg compared to 14.2 pmol/mg in patients without coronary artery disease (p = 0.019). This increase was also observed with stroke, dyslipidemia and metabolic syndrome with 2 times, 2.5 times and 3.5 times higher urinary 20-HETE/Cr ratios respectively in comparison to healthy individuals (Table 1).

Conflicting with these findings, the use of anti-hypertensive drugs, including beta blockers, angiotensin converting enzyme (ACE) inhibitors, or angiotensin receptor blockers (ARBs) resulted in a twofold increase in urinary 20-HETE/Cr ratio (p < 0.05) (Table 1). Similarly, insulin treatment increased urinary 20-HETE/Cr ratio (p < 0.001). (Table 1).

Urinary 20-HETE is a potential biomarker for kidney dysfunction

In our effort to investigate 20-HETE as a potential diagnostic and prognostic biomarker for DKD, we assessed the correlation between urinary 20-HETE/Cr ratio and standardized renal function tests. Blood urea nitrogen (BUN), creatinine, serum calcium, serum phosphorus, uric acid, transferrin levels, urine albumin, albumin to creatinine, and protein to creatinine ratios positively correlated with urinary 20-HETE/Cr ratio (p < 0.05). Conversely, a negative correlation was noted between urinary 20-HETE/Cr ratio and serum albumin, GFR, hematocrit, and urine creatinine levels (p < 0.05). These findings strongly suggest that urinary 20-HETE/Cr ratio **significantly increases with the severity of kidney disease and** is highly sensitive to detect renal dysfunction even when at early clinical stages (Table 2).

To further confirm the association between urinary 20-HETE levels and kidney function, a multivariate linear regression analysis was performed. In the multivariate regression analysis of all patients (182 subjects), out of the variables that were significantly associated with urinary 20-HETE/Cr ratio in the univariate analysis, family history of kidney disease, diabetes and an increased protein to creatinine ratio had an adjusted R-squared of 0.48 (Table 3). Since our model shows that urinary 20-HETE levels are positively correlated with kidney injury in patients with diabetes, and in order to indicate whether urinary 20-HETE/Cr ratios may serve as determinants for DKD, we performed a logistic regression analysis to identify the potential predictors of DKD. Our data shows that BMI, HbA1c, SBP, uric acid and urinary 20-HETE/Cr ratio may all serve as predictors of DKD. In fact, a 10-unit increase in urinary 20-HETE/Cr ratio was found to be associated with a 10-fold increased risk of developing DKD (OR = 1.175; p = 0.006) (Table 4). Intriguingly, when assessing the sensitivity and specificity of 20-HETE levels in detecting kidney injury in all subjects, we found that a urinary 20-HETE/Cr ratio of 6.4 pmol/mg had a sensitivity of 77.1% and a specificity of 78.6% in detecting kidney injury with an AUC of 0.84 (Fig. 2A). More importantly, a urinary 20-HETE/Cr ratio of 4.6 pmol/mg had a sensitivity of 82.2% and a specificity of 67.1% in detecting albuminuria with an AUC of 0.81 (Fig. 2B). Furthermore, the ROC curve for our model predicting DKD, which includes the variable urinary 20-HETE/Cr ratio, shows that the model had a sensitivity of 92.9 % and a specificity of 99.5 % in predicting kidney disease in patients with diabetes with an AUC of 0.97 (Fig. 2C)Table 5.

Discussion

This study investigates the potential role of 20-HETE as an early diagnostic and prognostic biomarker for DKD. Increasing evidence reveals that serum 20-HETE levels are strongly correlated with CKD and cardiovascular injury [17,23]. In one finding, Afshinnia *et al.* screened for 85 eicosanoid metabolites in serum of patients with CKD and reported a positive correlation between serum 20-HETE levels and CKD progression [17]. However, the association between urinary 20-HETE and DKD progression remain to be elucidated. To our knowledge, this is the first study to elucidate a positive correlation between urinary 20-HETE levels and diabetes-induced kidney complications. This study also shows a positive correlation between urinary 20-HETE levels and diabetes-induced microvascular, such as retinopathy, and macrovascular complications. Our findings identify a potential role of 20-HETE as a non-invasive prognostic and diagnostic marker for DKD and, to a larger extent, for micro- and macrovascular diabetic complications.

It is well documented that diabetes mellitus coexists with various co-morbidities. Hence, we investigated the correlation between diabetes-associated co-morbidities and urinary levels of 20-HETE. Our findings indicate that urinary 20-HETE levels increase with hypertension, coronary artery disease, stroke, dyslipidemia, and metabolic syndrome (Table 1). These findings can be explained by the predominant effect of 20-HETE on the vascular system, where it stimulates vasoconstriction, modulates endothelial function, and regulates angiogenesis and vascular remodeling [24]. A recent study has well described a positive correlation between the polymorphisms of CYP enzymes producing 20-HETE and systemic hypertension in humans [16]. Other studies report that 20-HETE modulates blood pressure through its action on the renin angiotensin system and on the Na^+/K^+ proton pump [25]. These studies show an increase in 20-HETE levels when vascular ACE expression and circulating angiotensin II levels are elevated, thus promoting vascular dysfunction [25,26]. Conversely, 20-HETE has a natriuretic property in tubules where it inhibits the $Na^+/K^+/2Cl^-$ cotransporter in the loop of Henle and prevents sodium reabsorption, resulting in a drop in blood pressure [25,26]. In line with our findings, the underlying mechanisms of 20-HETE-induced hypertension are attributed to renal implications.

One of the intriguing findings in our study was the negative correlation between urinary levels of 20-HETE and the use of anti-hypertensive medications. Our data show that ACE inhibitors and ARBs are associated with high urinary levels of 20-HETE (Table 1). This finding, despite its ambiguity, is in accordance with a previous study in which ACE inhibitors or ARBs are found to decrease blood pressure without changing 20-HETE levels in models of 20-HETE-driven hypertension [27]. The results suggest, as also described by Garcia *et al* [27], that the renin angiotensin system contributes to 20-HETE-mediated microvascular remodeling in hypertension and that 20-HETE-induced microvascular remodeling, independently from the rise blood pressure, does not fully rely on ACE activity in vascular endothelium.

Looking at the full spectrum, 20-HETE levels were found to be significantly associated with statins (Table 1). Our findings are in line with prior results from a clinical study in which patients with kidney failure and treated with statins had increased levels of urinary 20-HETE [28]. Furthermore, aspirin was associated with higher levels of 20-HETE, which can be explained by assessing the mechanism of action of aspirin. Aspirin inhibits COX enzymes limiting the synthesis of prostaglandins and thromboxane A2. By blocking the COX pathways, AA metabolism is shifted to the CYP450 pathway [29].

In our study, diabetes, diabetes duration, HbA1c and family history of diabetes all resulted in a significant elevation of urinary 20-HETE/Cr ratio, while no significant correlation was observed between obesity or BMI and urinary 20-HETE/Cr ratio. Moreover, diabetes-induced nephropathy and retinopathy increased urinary 20-HETE levels (Table 1). The increase in urinary 20-HETE levels with the duration of diabetes may suggest the implication of 20-HETE in an underlying mechanism for the development of diabetes-induced complications. Taken together, our results show

Table 2
Association of urinary 20-HETE/Cr ratio with patient laboratory tests and renal function tests.

Laboratory tests	n	Mean (pmol/l) ± SD	Urinary 20-HETE/Cr ratioMean (pmol/l) ± SD or R correlation factor	p value
Blood studies				
BUN (mmol/l)	179	7.3 ± 7.04	0.315	**<0.001**
Creatinine (umol/l)	181	97.91 ± 65.31	0.371	**<0.001**
GFR (ml/min)	180	80.1 ± 33.35	−0.304	**<0.001**
GFR categories (n = 180)	**G1 (67)**	67	9.7 ± 13.9	**<0.001**
	G2 (69)	69	14.3 ± 20.7	
	G3a (15)	15	25.1 ± 32.8	
	G3b (13)	13	17.2 ± 13.8	
	G4 (15)	15	39.1 ± 24.7	
	G5 (1)	1	77.7 ± 0	
Serum albumin (g/l)	178	43.92 ± 4.62	−0.239	**0.001**
Serum total protein (g/l)	174	71.45 ± 174.13	−0.06	0.430
Corrected serum calcium (mmol/l)	167	2.25 ± 0.12	0.176	0.023
Serum phosphorus (mmol/l)	167	1.32 ± 0.77	0.204	0.008
Serum sodium (mmol/l)	180	138.84 ± 2.84	−0.126	0.093
Serum potassium (mmol/l)	178	5.33 ± 10.09	−0.035	0.639
Serum chloride (mmol/l)	171	98.63 ± 14.64	−0.047	0.544
Serum bicarbonate (mmol/l)	172	26.6 ± 8.82	−0.001	0.988
Hemoglobin (gm/dl)	169	13.86 ± 6.87	−0.012	0.877
Hematocrit (%)	169	39.84 ± 5.4	−0.151	**0.05**
Platelets (*1000/cmm)	169	258.93 ± 78.26	0.086	0.265
WBC (1000/cmm)	169	10.14 ± 16.85	−0.05	0.521
Total bilirubin (umol/l)	166	9.52 ± 18.58	0.021	0.79
ALT (U/L)	165	23.98 ± 15.06	0.041	0.597
AST (U/L)	153	21.97 ± 10.96	−0.009	0.915
ALKP (U/L)	172	77.37 ± 30.25	0.140	0.067
TG (mmol/l)	175	1.92 ± 1.57	0.045	0.555
Total Cholesterol (mmol/l)	155	5.2 ± 7.34	0.027	0.735
LDL (mmol/l)	177	2.68 ± 0.92	−0.118	0.118
HDL (mmol/l)	177	1.17 ± 0.48	−0.077	0.31
Uric acid (mmol/l)	142	313.26 ± 113.04	0.153	**0.069**
Transferrin saturation (%)	144	23.05 ± 18.47	0.210	0.012
Renal Function Tests				
ACR (mg/mmol)	124	10.21 ± 20.5	0.337	**<0.001**
Protein to creatinine ratio (mg/mmol)	158	71.45 ± 174.13	0.268	**0.001**
Proteinuria (>=50 mg/mmol) (n = 158)	Yes	33	31.9 ± 27.3	**<0.001**
	No	126	11.7 ± 18.2	
Severity of albuminuria (n = 124)	Normal (<3mg/mmol)	79 (63.7%)	8.0 ± 17.1	**<0.001**
	Moderate (3–30 mg/mmol)	32 (25.8%)	23.7 ± 26.9	
	Severe (>30 mg/mmol)	13 (10.5%)	33.2 ± 26.1	

20-HETE, 20-hydroxyeicosatetraenoic acid; ACR, albumin to creatinine ratio; ALT, alanine aminotransferase; ALKP, alkaline phosphatase; AST, aspartate aminotransferase; BUN, blood urea nitrogen; GFR, glomerular filtration rate; HDL, high density lipoprotein; LDL, low density lipoprotein; TG, triglycerides; WBCs, white blood cells.

Table 3
Various statistical models testing the predictability of urinary 20-HETE/Cr ratio as a prognostic and diagnostic marker for diabetic kidney disease.

Models		All patients [a] n = 124		
Variables		**B (pmol/l)**	**CI**	**p value**
Socio-demographic	Family history of CKD	25.4	14.5–36.4	<0.001
Comorbidities	Diabetes Mellitus	15.1	7.6–22.6	<0.001
	Stroke	20.6	2.6–38.7	0.025
Medications	Anti-platelets	13.3	4.9–21.8	0.002
	Statin	−7.2	−14.8_0.38	0.06
Kidney function	Protein to creatinine ratio >50 mg/mmol	10.1	1.9–18.1	0.016
Blood test	Corrected calcium			
	Transferrin saturation	0.216	0.054–0.379	0.009
Adjusted R-squared		0.48		

[a] : Adjusted for age, hypertension, diabetes status, coronary artery disease, stroke, family history of diabetes, family history of CKD, dyslipidemia, metabolic syndrome, Beta blocker, ACEI and ARB use, antiplatelets (aspirin), statin, SBP, BUN, serum albumin, corrected calcium, phosphorus, transferrin saturation levels, proteinuria, severity of decrease in GFR.

that 20-HETE plays a key role in the etiology of diabetes and more importantly, as previously described by our team and by others, 20-HETE is involved in complications of diabetes [13,17,30-34]. Our findings highlight the importance of using urinary 20-HETE as both a diagnostic and prognostic biomarker for diabetes and its complications.

Despite limited contradictory studies showing that urinary 20-HETE levels are correlated with normal renal function and

Table 4
A multivariate analysis showing urinary 20-HETE/Cr ratio as one of the potential predictors of diabetic kidney disease.

Models	DKD [a] n = 76		
Variables	**OR**	**CI**	**p value**
BMI (kg/m2)	1.29	1.08–1.55	0.005
HbA1C (in percent)	3.6	1.09–11.89	0.035
SBP (mmHg)	1.07	0.993–1.165	0.075
Uric acid (mmol/l)	1.01	1.00–1.02	0.036
Urinary 20-HETE/Cr ratio (pmol/mg)	1.17	1.05–1.32	0.006
Adjusted R-squared	0.77		

[a] This model was adjusted for: age, duration of diabetes, HbA1c levels, stroke, ACE inhibitors and ARBs, antiplatelets, statin, insulin intake, BMI, oral anti-diabetics, urinary 20-HETE/Cr ratio, SBP, uric acid and diabetic retinopathy.

are significantly lower in patients with DKD [35], and that the downregulation of peroxisome proliferator-activated receptors (PPARs) and circulating levels of 20-HETE plays a key role in the pathogenesis of DKD in an experimental mice model of DKD [36], our data indicate that urinary 20-HETE levels are higher in patients with kidney injury and are positively correlated with renal function tests, including BUN, creatinine, uric acid, urine albumin, albumin to creatinine ratio and protein to creatinine ratio. These findings are further confirmed with a negative correlation with serum albumin, GFR, hematocrit and urine creatinine. More importantly, our results indicate that urinary 20-HETE levels increase significantly with the severity of kidney injury, making urinary 20-HETE a potential prognostic marker for the progression of DKD (Table 2). Moreover, in our study the association between increased urinary 20-HETE levels and DKD is maintained in a multivariate linear regression model (Table 3). A plausible explanation for our findings could be attributed to the role of 20-HETE metabolite as a major source of ROS production in kidney glomeruli and tubules [13,17,30-34,37,38]. Accordingly, increased ROS production was shown to induce podocyte and tubular apoptosis, leading to albuminuria and proteinuria [13]. In these same studies, inhibition of 20-HETE production in diabetic animals reduced proteinuria and prevented glomerular and tubular epithelial cell depletion, renal damage and albuminuria through a decrease in ROS production [13]. More importantly, when assessing the performance of our model in detecting DKD, we found that urinary 20-HETE/Cr ratio is one of the variables that allows for the detection of kidney disease in patients with diabetes with a sensitivity of 92.9%, a specificity of 99.5% and an AUC of 0.97. **(**Fig. 2C**).**

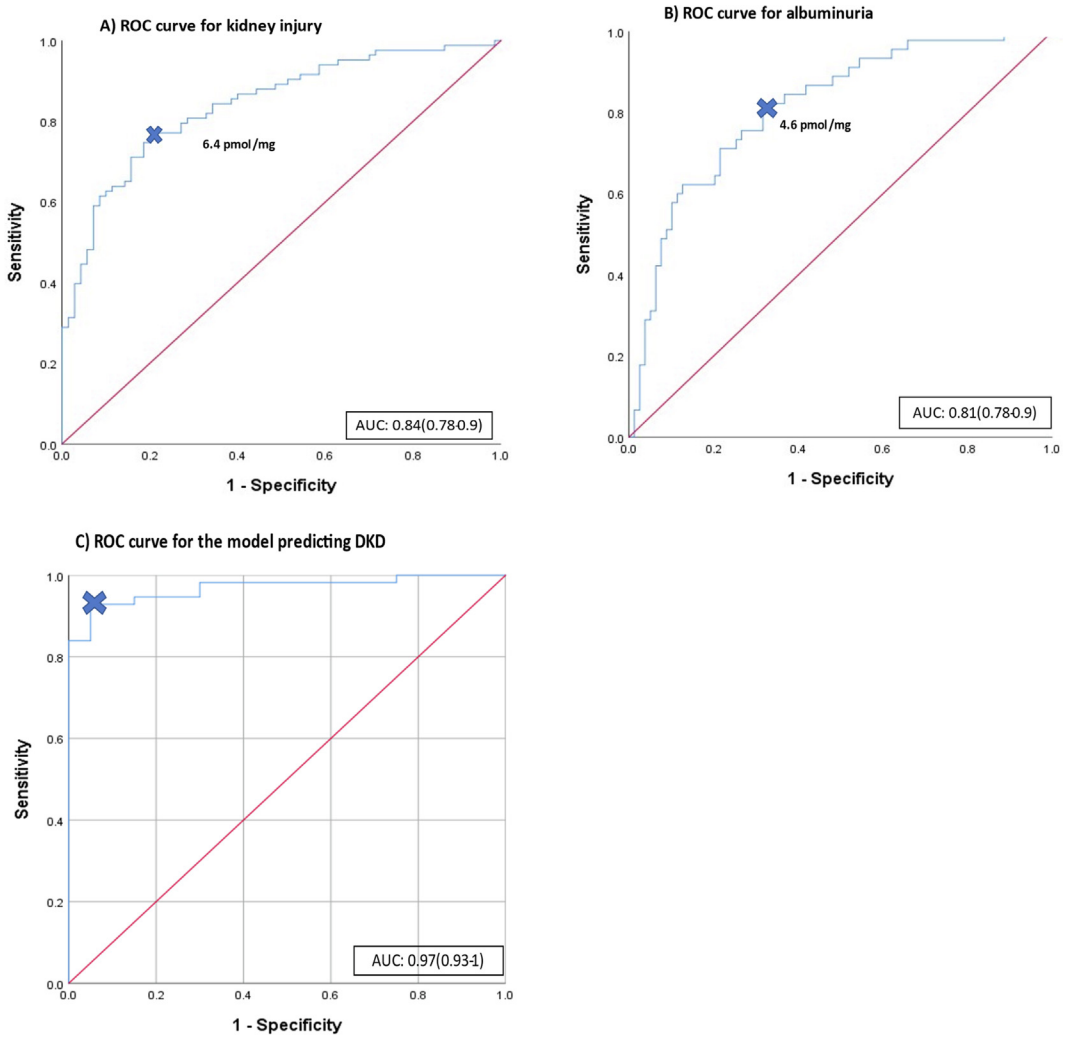

Fig. 2. 20-HETE as a Potential Diagnostic Tool, The illustrated ROC curves suggest 20-HETE as a potential diagnostic marker for kidney injury. Selection of 20-HETE levels of 6.4 pmol/mg (A) shows a sensitivity of 77.1.9%, a specificity of 78.6% and an AUC of 0.84 in detecting kidney injury in all subjects. B: In detecting albuminuria, 20-HETE levels of 4.6 pmol/mg had a sensitivity of 82.2% and a specificity of 67.1% with an AUC of 0.81. C: In diagnosing DKD, urinary 20-HETE/Cr ratio is one of the variables that allow for the detection of kidney disease in patients with diabetes with an AUC of 0.97, a sensitivity of 92.9% and a specificity of 99.5%.

Table 5
Results of ROC curves showing the diagnostic performance of urinary 20-HETE/Cr ratio in the diagnosis of kidney injury.

Graph	AUC	Confidence interval	P value	Youden index	sensitivity	specificity	20-HETE (pmol/mg)
Graph 1	0.84	0.78–0.90	<0.001	0.557	77.1%	78.6%	6.4
Graph 2	0.81	0.74–0.89	<0.001	0.493	82.2%	67.1%	4.6
Graph 3	0.97	0.93–1	<0.001	0.879	92.9%	99.5%	–

Our data sensitively suggest that urinary 20-HETE may serve as a potential predictor for the development and progression of diabetes-induced kidney disease. Our established ROC curves show adequate sensitivity and specificity, reflecting diagnostic accuracy. The cutoff points obtained from these curves vary between 4.6 pmol/mg and 6.4 pmol/mg. These levels are possible cut offs that need to be validated with further studies.

Notably, genetic predisposition has been proposed as an important factor for the development of DKD, further reinforcing our findings [39]. In accordance, we found a significant correlation between family history of CKD and 20-HETE levels, suggesting a hereditary component that modulates the synthesis of 20-HETE.

This study is a pilot study suggesting that the alteration in urinary 20-HETE/Cr ratio correlates with DKD. Nevertheless, it has limitations. Patients were recruited from a nephrology clinic, which led to a selection bias manifested by the high prevalence of DKD in the study sample. Although, the participants came from different regions, the sample is not representative of their respective regions, and the results cannot be extrapolated to the participants' respective countries. Also, the inclusion criteria were broad which might have affected the outcome. This study established an association between 20-HETE levels and DKD, but a cohort study of people with diabetes would be a better choice to reflect the true relationship between urinary 20-HETE/Cr ratio and DKD and to assess its predictive value.

Conclusion

Current screening tests for DKD mainly rely on monitoring urinary albumin excretion rates. However, by the time albuminuria is detectable, substantial tissue injury would have occurred [14]. If earlier detection of renal injury can be achieved, preventative therapeutic interventions might be conceived. Implementing earlier detection tests is anticipated to have major medical, financial and socioeconomic advantages for patients with diabetes. **Earlier detection can help overcome therapeutic inertia by achieving a timely medical intervention to preserve kidney function in patients with diabetes.** Our data show that urinary 20-HETE levels are associated with albuminuria and kidney injury and may serve as potential diagnostic and prognostic markers for DKD.

Compliance with Ethics Requirements

In this study, all procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008. Informed consent was obtained from all patients recruited in this study. (Hamad General Hospital, Doha, Qatar - IRB study no. 11313/11).

CRediT authorship contribution statement

Pamela Houeiss: Formal analysis, Investigation, Data curation, Writing – original draft, Visualization. **Rachel Njeim:** Investigation, Data curation, Writing – original draft, Visualization. **Hani Tamim:** Methodology, Formal analysis, Writing – review & editing. **Ahmed F. Hamdy:** Investigation, Data curation, Resources, Writing – review & editing. **Tanya S. Azar:** Validation, Writing – review & editing. **William S. Azar:** Validation, Writing – review & editing. **Mohamed Noureldein:** Validation, Writing – review & editing. **Youssef H. Zeidan:** Validation, Writing – review & editing. **Awad Rashid:** Methodology, Data curation, Resources, Writing – review & editing. **Sami T. Azar:** Methodology, Validation, Writing – review & editing. **Assaad A. Eid:** Conceptualization, Methodology, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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