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Using Shear Wave Elastography (SWE) for Early Non-Invasive Detection of Renal Fibrosis and its Correlation to Human Epididymis Protein 4 (HE4) Biomarker

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Abstract: Background: Assessment of chronic kidney disease is very difficult and is usually detected using renal biopsy which is an invasive procedure and may results in complications. A new image technique Short Wave Elastography (SWE) is now under development to measure kidney elasticity, and to assess the capability of detecting kidney diseases at earlier stages. Contemporary investigations have delineated Human Epididymis Protein 4 (HE4) as a progressive oncological biomarker instrumental in monitoring ovarian cancer recurrence among women. Concomitantly, enhanced expression of the HE4 gene has been observed in myofibroblasts implicated in fibrotic processes, indicating its prospective significance as a diagnostic marker for renal fibrosis. The aim of our study is assessment of (SWE) for measuring the degree of fibrosis in the kidney as noninvasive method for diagnosis of fibrosis in patients with chronic kidney disease (CKD) whether dialysis or pre-dialysis patients and measurement HE4 protein level in CKD patients and assessment of using it as a biomarker of renal fibrosis and correlation between HE4 level and degree of fibrosis in CKD patients. Methods: In this investigation, a cohort of ninety-three individuals was scrutinized, comprising sixty-two patients diagnosed with CKD. This patient cohort was stratified into two groups: thirty individuals receiving conservative pre-dialysis care and thirty-two undergoing hemodialysis. A control group consisting of thirty-one subjects, matched for age and sex with the patient cohort, was also included. Renal stiffness was assessed utilizing SWE imaging techniques, and levels of HE4 were quantitatively measured in the sera of all participants employing ELISA methodologies. Results: SWE exhibits proficiency in detecting renal fibrosis in CKD patients, although it fails to discern distinct CKD stages classified by GFR. Conversely, HE4 holds potential as a diagnostic biomarker for the assessment of fibrotic progression in CKD cohorts

Keywords: chronic kidney disease -renal fibrosis- Human epididymis protein 4 - Short Wave Elastography

Introduction

Chronic Kidney Disease (CKD) constitutes a significant etiological component contributing to the escalating epidemiological burden and represents a substantial global health challenge (1). Across a global populace of 6.4 billion, in excess of 500 million individuals are afflicted with various forms and stages of renal disorders. Projections indicate that by 2030, the prevalence of patients necessitating renal replacement therapies such as dialysis or transplantation due to kidney failure will exceed 2 million (2,3). In the year 2017 alone, CKD was responsible for approximately 1.2 million fatalities worldwide. The pathophysiological mechanisms underpinning CKD involve a complex interplay of capillary network rarefaction, destruction, and necrosis, compounded by chronic hypoxia and inflammation (4), which collectively drive the progression of CKD via parenchymal fibrosis. Irrespective of the initial etiology, renal tissue undergoes a cascade of processes that commence with the activation of resident renal cells. This is followed by the release of pro-inflammatory cytokines and the recruitment of inflammatory monocytes/macrophages and T cells to sites of injury. Should the damage persist, the glomerular and interstitial inflammatory infiltrates become activated, secreting an array of pro-fibrotic and inflammatory cytokines, reactive oxygen species (ROS), and other pathogenic molecules, thus exacerbating the disease process (5). "Ultimately, mesangial cells, tubular epithelial cells, and fibroblasts are activated, undergoing phenotypic transitions that culminate in the synthesis of numerous ECM proteins. Myofibroblasts are widely recognized as the principal effector cells responsible for the formation of renal fibrosis. Consequently, their activationn is considered a pivotal event in the pathogenesis of renal fibrosis (6,7). Myofibroblasts are frequently identified as activated or differentiated fibroblasts. These cells originate from a diverse set of precursors, including bone marrow-derived fibroblasts, tubular epithelial cells, endothelial cells, pericytes, and interstitial fibroblasts (5). Renal fibrosis represents the terminal phase of all advancing renal pathologies. It is typified by the relentless and excessive build-up of ECM components, myofibroblasts, and infiltrative inflammatory cells, leading to the profound disruption of the normal renal architecture and consequent progressive impairment of renal function (8). Renal fibrosis, an irreversible process, precipitates glomerular sclerosis, tubular atrophy or dilation, interstitial fibrosis, and a reduction in the glomerular and peritubular capillaries. These structural distortions, while discernible through traditional grayscale ultrasound imaging, elude precise quantification (9). Although percutaneous renal biopsy exhibits high diagnostic accuracy, its invasive nature often predisposes to complications (10). Consequently, there is a critical need for a reliable non-invasive modality to detect renal fibrosis in patients with CKD and to assess responses to treatment (11). In our research, we utilize Shear Wave Elastography (SWE), a non-invasive method based on Acoustic Radiation Force Impulse (ARFI) technology, for the detection of renal fibrosis. SWE, an innovative ultrasound technique, facilitates the non-invasive assessment of tissue stiffness. This quantitative approach employs focused acoustic energy pulses to generate minute tissue displacements, which in turn produce perpendicular shear waves. These waves are sonographically monitored as they traverse the tissue. Tissue stiffness is directly proportional to the velocities of these shear waves. Expressed in kilopascals (kPa) and derived from shear wave velocity, Young's modulus (YM) provides a quantifiable measure of tissue stiffness, with higher values indicating more extensive fibrosis (12,13).

Recent findings have highlighted Human Epididymis Protein 4 (HE4) as the most significantly upregulated gene within fibrosis-associated myofibroblasts, suggesting its potential as a diagnostic biomarker for renal fibrosis (14).

Human Epididymis Secretory Protein 4 (HE4, also known as WFDC2), a glycoprotein characterized by N-linked glycosylation, is encoded by a locus on chromosome 20q12-13.1. Initially isolated as a unique transcript predominantly expressed within the epididymal region and implicated in the maturation processes of spermatozoa (15), subsequent research has elucidated its presence in additional anatomical sites such as the respiratory tract and oral cavity. Within these locales, HE4 is postulated to play a critical role in the orchestration of innate immune responses and may significantly influence the oncogenic pathways leading to lung adenocarcinoma (16). Furthermore, elevated expressions of HE4 have been consistently reported in the serum of ovarian cancer patients, manifesting notably even in the incipient stages of the disease, with a

pronounced prevalence in serous and endometrioid subtypes of epithelial ovarian cancer (17). Multiple investigative collectives have corroborated the efficacy of HE4 as a serum biomarker for diagnosing epithelial ovarian cancer (18). In addition, this biomarker received endorsement from the United States Food and Drug Administration (FDA) in 2009 as a reliable indicator for tracking the recurrence or advancement of the disease. In a more recent development, Le Bleu et al. (2013) delineated the role of HE4 as an effector in renal fibrosis, attributing its function to its inherent properties as a protease inhibitor capable of curtailing the enzymatic activity of multiple proteases, including serine proteases and matrix metalloproteinases. This inhibition is particularly significant in the context of preventing the degradation of type I collagen (19,20). In our investigation, we endeavored to quantify HE4 levels in patients afflicted with CKD and explored its association with renal fibrosis, as detected through SWE. Additionally, we assessed its utility as a potential biomarker for renal fibrosis.

Aim of work

Assessment of using (SEW) for measuring degree of fibrosis in the kidney as a noninvasive method for diagnosis of fibrosis in patients with chronic kidney disease (CKD) whether dialysis or pre-dialysis patients and its sensitivity. Measuring HE4 protein level in CKD patients and assessment of using it as a biomarker of renal fibrosis.

Methodology

Ethics approval

From Research Ethics Committee (REC), Theodor Bilharz Research Institute (TBRI) (FWA # 000019609) From 30 may2023. All participants signed a written informed consent.

Subjects

In the conducted research, a total of 93 subjects were incorporated, with 62 individuals identified as suffering from CKD. These participants were further segmented into two subsets: 30 patients on conservative treatment prior to dialysis and 32 undergoing regular hemodialysis. A control cohort consisting of 31 age- and sex-matched healthy volunteers was also assembled for comparative analysis. All individuals were sourced from the Nephrology Department and Hemodialysis Unit at Theodor Bilharz Research Institute, where they were subjected to a series of assessments:

1) **Full history taking**
2) **Full clinical examination**

3) Routine laboratory investigations:

Peripheral blood samples were procured from all participants via venous puncture and collected into vacutainer tubes designated for serum extraction. These samples were then permitted to clot at room temperature for a duration of one hour prior to undergoing centrifugation. Supernatant was then stored at -80°C till time of the assay. Routine kidney function tests were assayed in by standard biochemical laboratory methods; in the form of serum urea, creatinine, calcium (total), and phosphorus as well as glucose. The assays were conducted using an AU 480 chemistry analyzer from Beckman Coulter, situated within the clinical chemistry department of TBRI Hospital. The estimated glomerular filtration rate (eGFR) was computed utilizing the Modification of Diet in Renal Disease (MDRD) formula (21). Comprehensive assessments including a complete blood count (CBC), as well as measurements of serum urea, serum creatinine, and serum levels of calcium and phosphorus, were performed.

4) Special laboratory investigations in the form of: assessment of HE4 level in male patients and female patents after exclusion of ovarian cancer:

The measurement of Human HE4 (epididymal protein 4) concentrations in human serum was conducted utilizing the Elabscience® Human HE4 ELISA Kit (Catalog No: E-EL-H5433, USA), which employs a Sandwich-ELISA approach. The assay's microplate was pre-coated with specific antibodies that selectively bind to Human HE4. Into these antigen-affinitive wells, both analytical samples and calibration standards were deposited, followed by the administration of a targeted antibody designed to specifically interact with Human HE4. Subsequent to initial sample and standard additions, each designated well received a biotinylated detection

antibody specific to Human HE4 followed by an Avidin-Horseradish Peroxidase (HRP) conjugate, initiating an incubation phase. After this period, a thorough washing procedure was applied to remove non-specifically bound substances. A substrate solution was then introduced to each well, producing a blue color in wells containing Human HE4, the biotinylated detection antibody, and the Avidin-HRP complex. Termination of the enzymatic reaction was achieved by adding a stop solution, which shifted the color from blue to yellow. OD for each well was precisely quantified using spectrophotometric methods at a wavelength of 450 nm ±2 nm. The concentration of Human HE4 in the samples was accurately assessed by correlating the OD readings with a pre-established standard curve (22).

5) Conventional ultrasound and point Shear Wave Elastography examinations:

The ultrasound examinations were conducted using an Aplio 500 Ultrasound machine equipped with LASTPQ software and a convex probe by two seasoned ultrasonographers. Patients were positioned in either the supine or lateral decubitus position to optimize renal visualization. A standard conventional ultrasound scan was performed on both kidneys. During this, the renal length was measured along the coronal plane as the maximum distance between the superior and inferior poles. Kidney depth was determined by measuring the distance from the skin surface to the kidney (23). The transducer was oriented perpendicular to the renal capsule, and regions of interest (ROIs) were strategically placed within the cortex, carefully avoiding renal pyramids and blood vessels to ensure inclusion of only cortical tissue, while aligning the ROI as parallel as possible to the pyramids (24). The Young's Modulus (YM) of the kidney cortex was assessed at end-inspiration, with patients instructed to hold their breath. Measurements were taken at the mid kidney and at both poles. A minimum of five effective readings were captured for each assessment, and the average value was computed.

Statistical analysis

Data encoding and entry were conducted using the Statistical Package for the Social Sciences (SPSS) software, Version 28, provided by IBM Corp., Armonk, NY, USA. For quantitative datasets, aggregation and summarization were executed utilizing statistical measures such as mean, standard deviation, median, and the range (minimum and maximum). In contrast, categorical datasets were analyzed through the computation of frequencies (counts) and their corresponding relative frequencies (percentages). Comparative statistical analyses between quantitative groups were performed employing the non-parametric Mann-Whitney U test for dichotomous group comparisons and the Kruskal-Wallis test for scenarios involving three or more groups, with subsequent multiple comparisons facilitated by Dunn's test to identify significant variances across groups. For the comparison of serial measurements within each patient, the non-parametric Friedman test and Wilcoxon signed-rank test were utilized. For comparing categorical data between groups, the Chi-square (χ^2) test was performed. When the expected frequency was less than 5, the Fisher Exact test was employed instead. Correlations between quantitative variables were determined using the Spearman correlation coefficient. Statistical significance was established at p-values < 0.05. A ROC curve was constructed, and area under curve analysis was performed to determine the optimal cutoff value of HE4 and elastography for detecting CKD and fibrosis. P-values less than 0.05 were considered statistically significant.

Results:

The demographic data showed that, the total number of subjects was (92 subjects) divided as; in dialysis group; (32 subjects), pre-dialysis group (30 subjects), and control group (31 subjects).In dialysis group 18 (56.25%) were males and 14 (43.75%) were females, while in pre-dialysis group; 17 (56.67%) were males and 13 (43.33%) were females, in control group 16 (53.33%) were males and 15 (46.67%) were females. The age in dialysis group was (mean53.53 ±7.71SD), in pre-dialysis group(mean 52.90 ± 9.81SD), in control group (mean51.19 ± 14.08SD), with no significant differences among the groups (p= 0.679). The incidence of DM was significantly more prevalent in patients as compared to the controlgroup (Table1)

Table 1: Demographic data and incidence of DM in the studied groups

		Dialysis (n=32)		pre-dialysis (n=30)		Control (n=31)		P value
		Count	%	Count	%	Count	%	
Sex	**Male**	18	56.25%	17	56.67%	16	53.33%	0.906
	Female	14	43.75%	13	43.33%	15	46.67%	
DM	**Diabetic**	11	34.4%	11	36.7%	0	0.0%	0.001
	Not diabetic	21	65.6%	19	63.3%	31	100.0%	
Age		**Mean** 53.53	**SD** 7.71	**Mean** 52.90	**SD** 9.81	**Mean** 51.19	**SD** 14.08	0.679

We found that serum urea, creatinine, hemoglobin, calcium, e GFR and degree of fibrosis differed significantly among the studied groups. Post-hoc pairwise analysis revealed that serum urea, creatinine and degree of fibrosis were significantly higher in dialysis and pre-dialysis patients as compared to control group, also hemoglobin serum calcium and e GFR were significantly lower in dialysis and pre-dialysis patients as compared to control group (Table 2).

Table 2: Laboratory parameters, age degree of renal fibrosis in the study population

	Dialysis		pre-dialysis		control		P value
	Mean	SD	Mean	SD	Mean	SD	
Urea	161.56	41.64	156.59	41.27	40.29	3.49	< 0.001
S.creat.	9.50	2.83	3.77	1.41	0.75	0.22	< 0.001
Hb%	9.44	1.54	9.39	0.78	12.39	0.75	< 0.001
Ca	8.85	0.89	8.90	0.40	9.45	0.40	< 0.001
P	4.88	1.82	4.20	0.62	4.68	0.41	0.061
eGFR	19.50	3.75	40.80	6.24	112.14	46.67	< 0.001
Fibrosis Rt kidney	10.88	2.23	8.41	1.31	2.95	0.50	< 0.001
Fibrosis Lt kidney	10.83	2.36	8.37	1.32	2.71	0.65	< 0.001

S.creat; serum creatinine, Hb; hemoglobin, Ca; calcium, P; phosphorus, eGFR; estimated glomerular filtration rate, Rt; right, Lt; left. *Statistically significant at p<0.05 was considered significant*

SWE in CKD patients:

Themeanvalues ofSWE (kPa)in pre-dialysis patients in right kidney were(8.41)while in left kidney were (8.37).The mean SWE values in CKD stages/dialysis patient in right kidney were(10.88) while in left kidney is (10.83). The mean values of SWing control group were (2.95) in the right kidney,while of left kidney is (2.71). There were no statistical significance differences of SWE values between the right and left kidney however in some patients we found that the SWE values differ among different areas of the same kidney (fig1-4). Despite

the significant increase in SWE values correlating with the progression of CKD stages, the statistical significance of the differences was more pronounced between the control group and the dialysis group than between the pre-dialysis and dialysis groups.

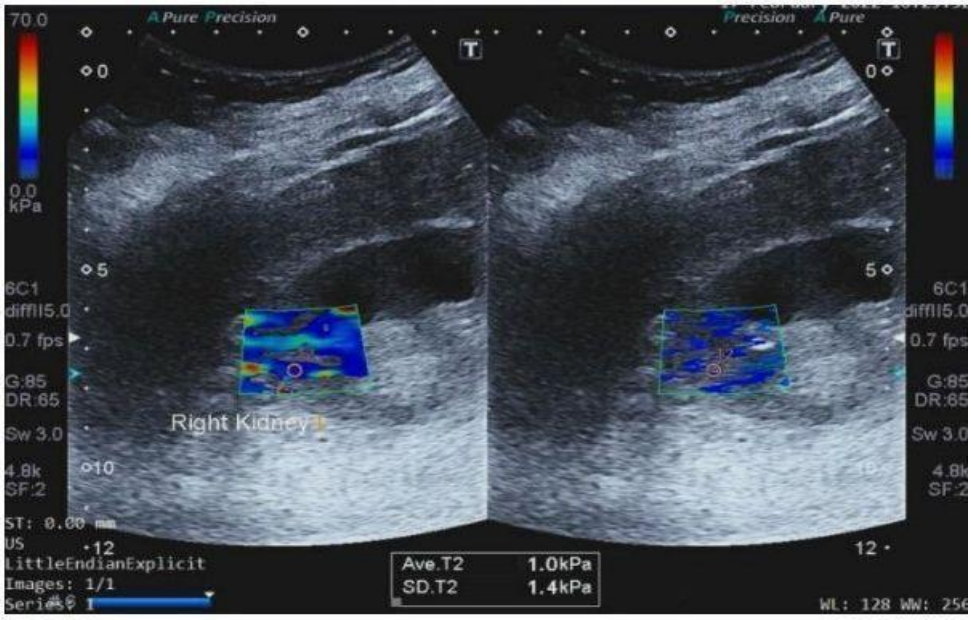


Figure 1: normal kidney from control group showing normal size, cortical thickness, echogenicity and SWE values (right kidney stiffness YM mean value=1.0 kpa).

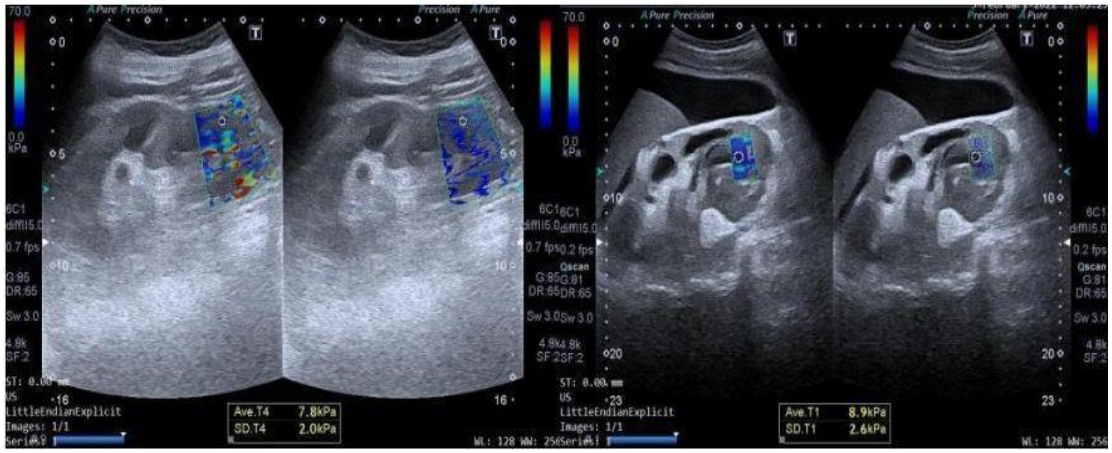


Figure 2: two different patients from the pre-dialysis group showing increased cortical echogenicity, and degree of renal stiffness (mean YM values =7.8 and 8.9 kpa).

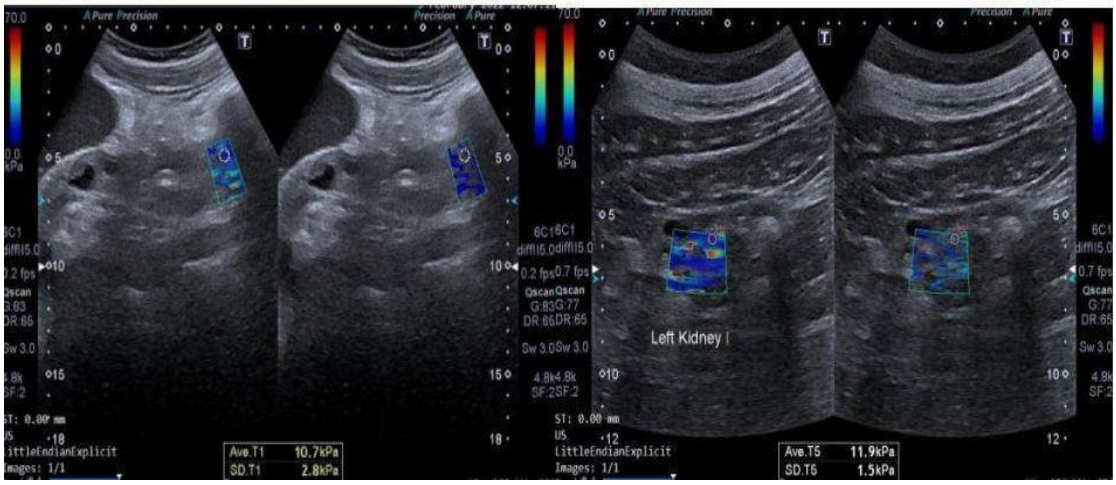


Figure 3: two different patients from the dialysis group showing relatively small sized kidneys with increased echogenicity and reduced cortico-medullary differentiation. Values of kidney stiffness are raised (mean YM values are 10.7 11.9 kpa).

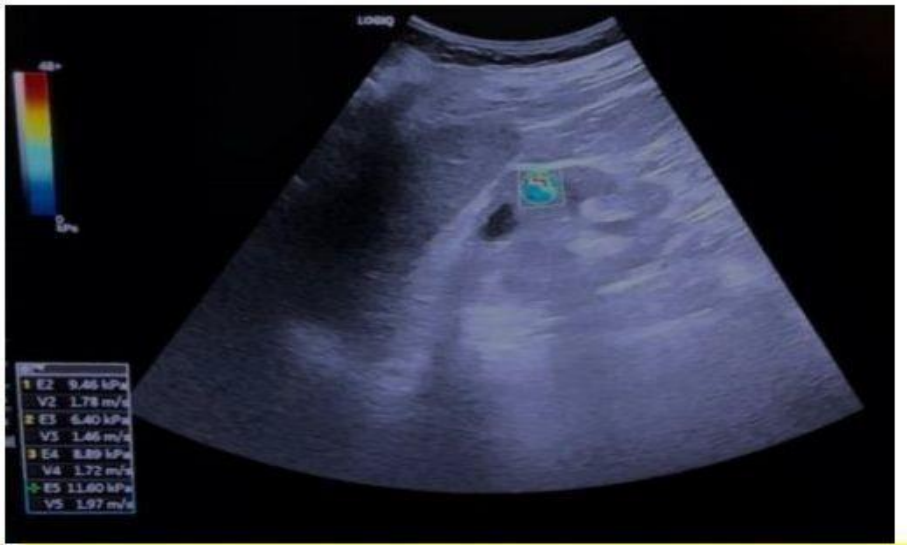


Figure 4: shows different SWE values at different parts of the same kidney.

In some patients we found that the SWE values differ among different areas of the same kidney (fig 4). Despite that the SWE values increased significantly with the increase of the CKD stage. There was no significant difference between the degree of fibrosis of right and left kidneys in all groups.

Post-hoc pairwise analysis of laboratory data and renal fibrosis showed a significant difference between both dialysis and pre-dialysis patients compared to the control group in urea, creat, Hb%, eGFR, fibrosis in Rt kidney and fibrosis in left kidney ($P< 0.001$), in Ca ($P<0.001$& 0.002 respectively) and a significant difference between dialysis and pre-dialysis in serum creat., e GFR ,fibrosis in Rt kidney and fibrosis in Lt kidney ($P<0.001$,0.008,0.001 and 0.001 respectively) Table 3.

Table 3: Post-hoc pairwise analysis of laboratory data and renal fibrosis by elastography in the study population:

		Dialysis	pre-dialysis	control
urea	**Dialysis**		1.000	< 0.001
	pre-dialysis	1.000		< 0.001
	control	< 0.001	< 0.001	
S.creat.	**Dialysis**		< 0.001	< 0.001
	pre-dialysis	< 0.001		< 0.001
	control	< 0.001	< 0.001	
Hb%	**Dialysis**		1.000	< 0.001
	pre-dialysis	1.000		< 0.001
	control	< 0.001	< 0.001	
Ca	**Dialysis**		1.000	0.001
	pre-dialysis	1.000		0.002
	control	0.001	0.002	
eGFR	**Dialysis**		0.008	< 0.001
	pre-dialysis	0.008		< 0.001
	control	< 0.001	< 0.001	
Fibrosis Rt kidney	**Dialysis**		< 0.001	< 0.001
	pre-dialysis	< 0.001		< 0.001
	control	< 0.001	< 0.001	
Fibrosis Lt kidney	**Dialysis**		< 0.001	< 0.001
	pre-dialysis	< 0.001		< 0.001
	control	< 0.001	< 0.001	

S.creat; serum creatinine, Hb; hemoglobin, Ca; calcium, P; phosphorus, eGFR; estimated glomerular filtration rate, Rt; right, Lt; left. Statistically significant at p<0.05 was considered significant

We measured the degree of fibrosis in both Rt and Lt kidney using Short Wave Elastography in all the groups, and we found a significant difference between the 3 groups with higher degree of fibrosis in dialysis patients compared to pre dialysis patients and to control. (P < 0.001). The degree of fibrosis was positively correlated to kidney function and estimated GFR. (P < 0.001). HE4 level was significantly elevated in chronic renal failure patients as compared to the control group, (P < 0.001). Table 4 and Figure 5).

Table 4: HE4 level among the studied groups

	Dialysis			pre-dialysis			Control			
	Median	**1st quartile**	**3rd quartile**	**Median**	**1st quartile**	**3rd quartile**	**Median**	**1st quartile**	**3rd quartile**	**P value**
HE4 (pmol /l)	13400.00	8750.00	18950.00	1880.00	988.00	4045.00	28.00	20.00	45.00	< 0.001

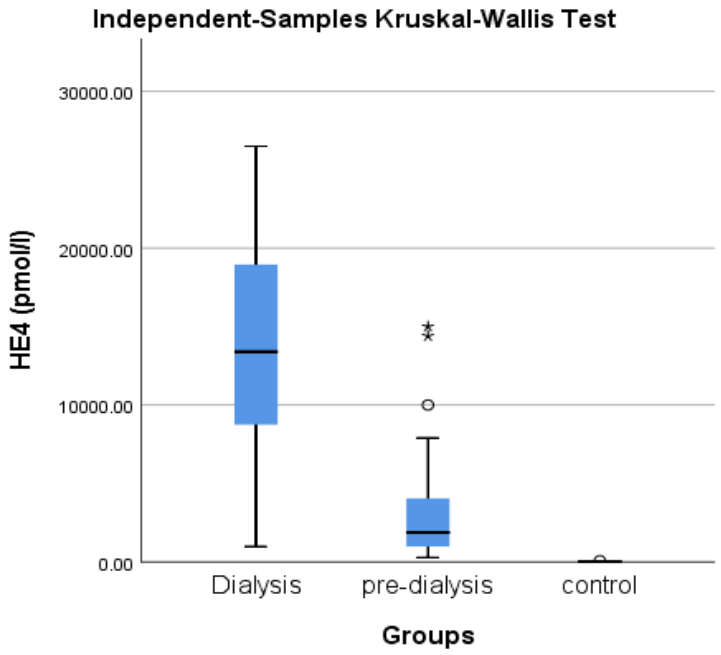


Figure 5: HE4 levels in the studied groups

When we compared HE4 level in both pre-dialysis and dialysis patients we found that HE4 level was significantly elevated in dialysis patients as compared to pre-dialysis patients (P=0.002). Table 5)

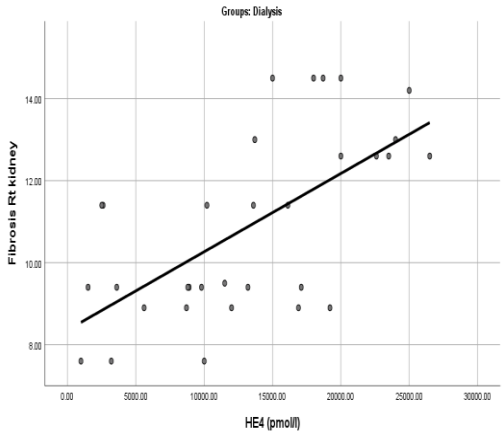
Table 5: Post-hoc pair wise analysis for HE4 in the study population

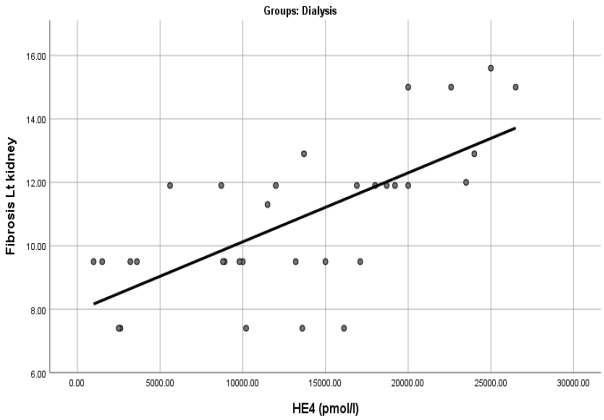
Groups	**P value**
control-pre-dialysis	< 0.001
control-Dialysis	< 0.001
pre-dialysis-Dialysis	0.002

As regarding correlation between HE4 and Degree of Fibrosis in the 3 different groups, we found highly positive correlation between with HE4 and degree of fibrosis in the dialysis group in both Rt and Lt kidney (r=0.621, P < 0.001), (r= 0.686P < 0.001) respectively. as shown in table 6, and figure 6,7.Also, we found a significant positive correlation between HE4 and degree of fibrosis in pre-dialysis group, (r=0.601, P < 0.001,) for right kidney, but not forLt kidney. Table 7 and Figure 8. This may be related to the different microvascular state of both kidneys.

Table 6: correlation between HE4 and degree of fibrosis by elastography in dialysis group

Dialysis		**Fibrosis Rt kidney**	**Fibrosis Lt kidney**
HE4 (pmol/l)	**Correlation Coefficient**	0.621	0.686
	P value	< 0.001	< 0.001
	N	32	32





Correlation between HE4

Figure 6: correlation between HE4 level and degrefibrosis in right kidney in the dialysis groups

Figure 7: correlation between HE4 level and degree of fibrosis in left kidney in the dialysis groups

Table 7: correlation between HE4 and degree of fibrosis by elastography in pre-dialysis group

pre-dialysis		**Fibrosis Rt kidney**	**Fibrosis Lt kidney**
HE4 (pmol/l)	**Correlation Coefficient**	0.601	0.191
	P value	< 0.001	0.313
	N	30	30

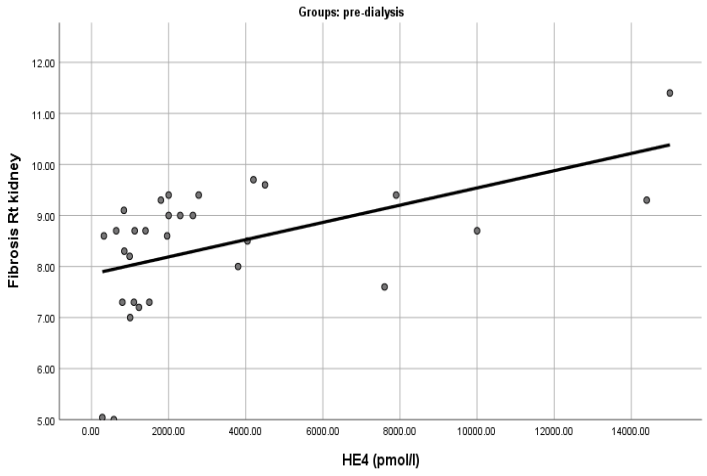


Figure 8: correlation between HE4 level and degree of fibrosis in right kidney in the pre- dialysis group

We investigated the correlations between renal fibrosis and other parameters in the dialysis patients, we found a significant negative correlation of Hb levels and the degree of fibrosis. No correlation was found with other parameters. Table 8.

Table 8: Correlation between HE4 level and Degree of fibrosis and other parameters (Age, gender, urea, creat, Ca, P, eGFR) in dialysis group

Dialysis		**Fibrosis Rt kidney**	**Fibrosis Lt kidney**	**HE4 (pmol/l)**
Age	**Correlation Coefficient**	0.003	0.025	0.048
	P value	0.989	0.892	0.796
	N	32	32	32
urea	**Correlation Coefficient**	0.139	0.266	0.256
	P value	0.447	0.141	0.158
	N	32	32	32
creat.	**Correlation Coefficient**	0.155	0.222	0.316
	P value	0.396	0.221	0.078
	N	32	32	32
Hb%	**Correlation Coefficient**	0.048	-0.014-	0.081
	P value	0.796	0.941	0.661
	N	32	32	32
Ca	**Correlation Coefficient**	-0.269-	-0.221-	-0.194-
	P value	0.137	0.224	0.288
	N	32	32	32
P	**Correlation Coefficient**	-0.066-	0.130	0.166
	P value	0.718	0.477	0.364
	N	32	32	32
eGFR	**Correlation Coefficient**	0.160	0.277	0.244
	P value	0.382	0.125	0.179
	N	32	32	32

S.creat; serum creatinine, Hb; hemoglobin, Ca; calcium, P; phosphorus, eGFR; estimated glomerular filtration rate. Statistically significant at p<0.05 was considered significant

We investigated the correlations between renal fibrosis and other parameters in the pre-dialysis patients we found a significant negative correlation between serum calcium level and the degree of fibrosis as the calcium level is lower the renal fibrosis is higher (P= 0.008, r= -0.47), with no significant correlation in dialysis patients. Table 9, Figure 9. Also we found a significant positive correlation between serum phosphorus level and the HE4 level in the pre-dialysis group (P= 0.017, r= 0.433). Table 9 and Figure 10 .

Table 9: Correlation between HE4 level and Degree of fibrosis and other parameters (Age, gender, urea, creat, Ca, P, eGFR) in pre-dialysis group

pre-dialysis		**Fibrosis Rt kidney**	**Fibrosis Lt kidney**	**HE4 (pmol/l)**
Age	**Correlation Coefficient**	-0.323-	0.211	0.000
	P value	0.082	0.262	0.998
	N	30	30	30
Urea	**Correlation Coefficient**	-0.071-	0.022	0.123
	P value	0.711	0.908	0.518
	N	30	30	30
S.creat.	**Correlation Coefficient**	-0.267-	0.081	-0.074-
	P value	0.154	0.669	0.697
	N	30	30	30
Hb%	**Correlation Coefficient**	0.218	-0.255-	0.045
	P value	0.247	0.173	0.813
	N	30	30	30
Ca	**Correlation Coefficient**	-0.135-	-0.477-	-0.039-
	P value	0.477	0.008	0.838
	N	30	30	30
P	**Correlation Coefficient**	0.171	0.080	0.433
	P value	0.365	0.673	0.017
	N	30	30	30
eGFR	**Correlation Coefficient**	0.004	-0.058-	-0.123-
	P value	0.982	0.761	0.518
	N	30	30	30

S.creat; serum creatinine, Hb; hemoglobin, Ca; calcium, P; phosphorus, eGFR; estimated glomerular filtration rate. Statistically significant at $p<0.05$ was considered significant

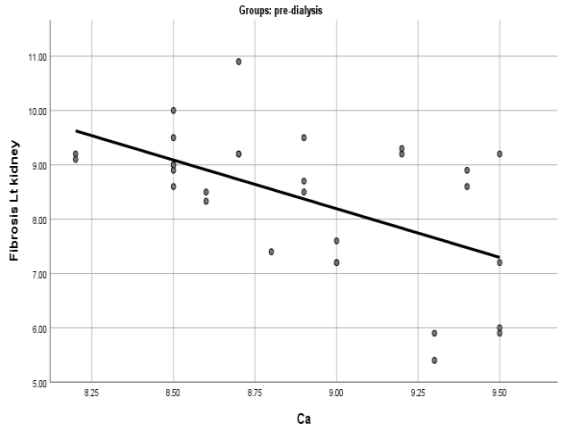


Figure 9: correlation between the degree of fibrosis in left kidney and calcium in the pre- dialysis group

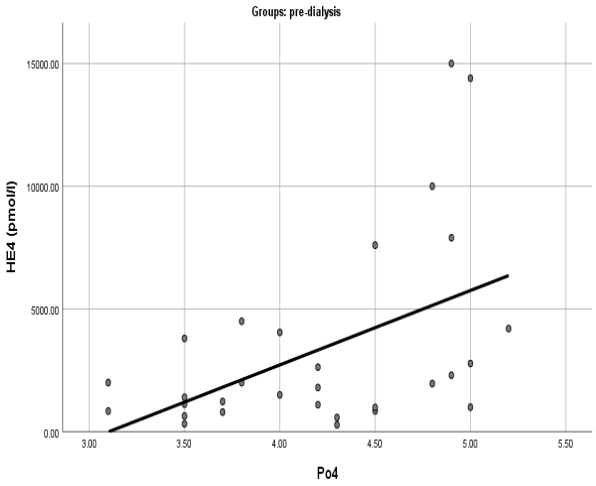


Figure 10: correlation between the HE4 level and phosphorus in the pre- dialysis group

There is no significant difference between males and females as regarding HE4 level in CKD patients, but as regarding renal fibrosis, we observed a higher degree of fibrosis in males more than females in dialysis (p=0.022) but not in pre-dialysis patients. Table 10.

Table 10: comparison of the degree of fibrosis by SWE in dialysis group between males and females

Dialysis	**Sex**				
	Male		**Female**		**P value**
	Mean	**Standard Deviation**	**Mean**	**Standard Deviation**	
Fibrosis Rt kidney	11.00	2.39	10.73	2.10	0.739
Fibrosis Lt kidney	11.65	2.41	9.76	1.88	0.022

We analyzed the elevation of HE4 level and degree of fibrosis in diabetic and non –diabetic patients; we found a significant positive correlation between both HE4 level and diabetes in pre-dialysis patients. This may be related to microvascular injury in these patients. Table 11 and Figure11.

Table 11: comparison of the level of HE4 in dialysis and pre-dialysis groups between diabetics and non-diabetics:

pre-dialysis	**DM**						
	Diabetic			**Not**			**P value**
	Median	**Percentile 25**	**Percentile 75**	**Median**	**Percentile 25**	**Percentile 75**	
HE4 (pmol/l)	2780.00	1960.00	10000.00	1100.00	800.00	2300.00	0.003
Dialysis							
HE4 (pmol/l)	16900.00	10000.00	20000.00	11500.00	5600.00	18000.00	0.208

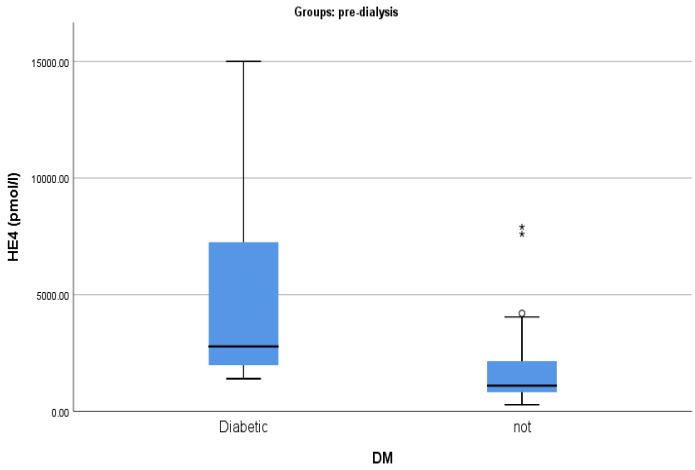


Figure 11: comparison of the level of HE4in dialysis and pre-dialysis groups between diabetics and non diabetics

Receiver operating characteristic curve (ROC curve) for assessment of diagnostic sensitivity and specificity of both HE4 and SWE:

ROC curve was performed for both HE4 and SWE to evaluate their diagnostic performance in the detection of CKD stage and renal fibrosis. We found that serum HE4 at cutoff value of 8300 pmol/l could discriminate between pre-dialysis group (CKD stage 2-3) and dialysis group (CKD stage 4-5) and the degree of fibrosis with AUC= 0.884, sensitivity of 78.1% and specificity of 90%, p <0.001, table 12, figure 12.In our study the cut-off value for predicting kidney fibrosis in renal dialysis patient was 9.35 kPa with 90.6% sensitivity and 86.7% specificity and AUC was 0.915 respectively. Table 12 and Figure 12.

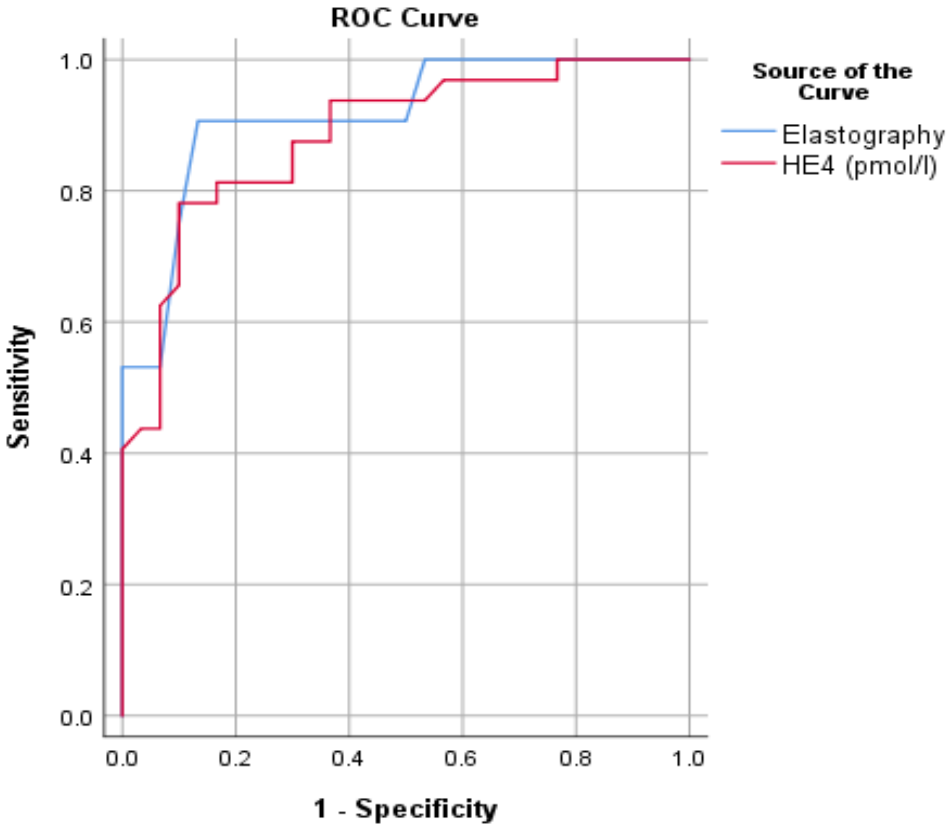


Figure 12: ROC curve for HhgfghfhfgE4 and SWE

Table 12: ROC curve cutoff values, AUC for HE4 and SWE

	Area under curve	P value	95% Confidence Interval		Cutoff value	Sensitivity %	Specificity %
			Lower Bound	Upper Bound			
HE4 (pmol/l)	0.884	**<0.001**	0.801	0.967	8300	**78.1**	**90**
Elastography	0.915	**<0.001**	0.846	0.985	9.35	**90.6**	**86.7**

Discussion:

Human epididymis protein-4 (HE-4) is a proteinase inhibitor that was initially linked to the epithelial cells of the epididymal duct. The results of many studies showed that HE4 was highly expressed in cancer tissues, especially ovarian cancer and it is commonly used as a diagnostic biomarker for ovarian cancer, as it can be measured easily in serum using ELISA technique (25). Prior investigations have demonstrated an upsurge in serum HE4 concentrations among individuals afflicted with CKD (26). Renal elastography, a novel noninvasive diagnostic tool, facilitates the assessment of tissue fibrosis, thereby eliminating the necessity for invasive biopsy procedures. This technique quantifies tissue stiffness or elasticity via ultrasound, based on the foundational understanding that neoplastic and fibrotic tissues manifest distinct elastic characteristics when compared to their normal tissue counterparts. (27).

In our study, we investigated the correlation between the degree of fibrosis detected by elastography and the level of serum HE-4 in dialysis and pre-dialysis patients as compared to healthy controls after exclusion of ovarian cancer in female participants using ultrasound.

We measured the degree of fibrosis in both kidneys using Short Wave Elastography in patients and controls and we found that the degree of fibrosis was significantly higher in dialysis patients as compared to pre dialysis patients and to normal control. The degree of fibrosis was positively correlated to kidney function and estimated GFR.

As renal fibrosis advances, kidney function deteriorates progressively until ESRD is achieved, compelling patients to commence dialysis. This decline is primarily due to activated renal fibroblasts precipitating extracellular matrix proteins, resulting in reduced kidney functionality (28). This phenomenon was substantiated in our study, where we identified a significant negative correlation between estimated GFR and the extent of renal fibrosis.

In our investigation, it was determined that serum levels of HE4 were notably higher in individuals experiencing chronic renal failure—both those undergoing dialysis and those in the pre-dialysis phase—when compared to a normative control group. This enhancement in serum HE4 has been corroborated by recent studies, which suggest a link between elevated HE4 levels and compromised renal function in patients diagnosed with CKD or AKI (29). The propensity for HE4 levels to increase in CKD can be ascribed to the diminutive molecular size of HE4, which naturally facilitates its passage through the glomerular filtration barrier. As renal fibrosis intensifies and the GFR diminishes, the clearance of HE4 is impeded, culminating in its accumulation within the serum. Additionally, there is a marked increase in HE4 mRNA expression within CKD conditions, which fosters augmented HE4 synthesis in fibrotic renal tissues (30). This regulatory enhancement in HE4 gene expression is precipitated by a sequence of cellular transformations within CKD, wherein renal cells proliferate and morph into myoblasts. This transformation is critical as the HE4 gene undergoes significant upregulation in these myoblasts, leading to an increase in HE4 production and secretion. HE4's role as a protease inhibitor is crucial, as it hinders the breakdown of collagen I by obstructing the activity of numerous serine proteases and matrix metalloproteinases, thereby facilitating the accumulation of type I collagen in the renal matrix and further promoting fibrotic processes (31)—a phenomenon validated through our empirical findings.

In evaluating the correlation between HE4 levels and the extent of fibrosis among three differentiated groups, we identified a notably positive correlation specifically within the dialysis cohort. This observation is consistent with findings from a study conducted by Song et al., 2023 (30), which included a diverse cohort of 187 adult patients with CKD, alongside 32 patients with AKI who had pre-existing CKD at stages 3–5, 59 patients at CKD stages 4–5, and 96 patients at CKD stages 1–3. Song et al. measured serum HE4 concentrations and discovered not only an elevation in these levels among CKD patients but also a positive correlation of HE4 with the disease's severity and the patients' prognoses.

Similarly, Yan et al., (2023), in their study which included 80 pathologically confirmed ovarian cancer patients, 641 CKD patients, and 2661 healthy controls, they found that CKD patients had the highest levels of serum HE-4, as compared to ovarian cancer patients and healthy controls (31). HE-4 levels were significantly correlated with CKD progression, which come in accordance with our results.

Additionally, Wan et al. (2016) demonstrated that elevated HE4 levels were indicative of more severe renal fibrosis. Their correlation analysis revealed a significant relationship between HE4 levels and the degree of renal fibrosis, with r of 0.938. AUC for HE4 was 0.9, surpassing that of serum creatinine, thereby underscoring the potential of HE4 as a superior biomarker for assessing renal fibrosis (32).

Another study performed by Lebleu et al., (2013) found that the administration of HE4-neutralizing antibodies led to inhibition of renal fibrosis in three different mouse models of renal disease. Which indicated that HE4 is a potential biomarker of renal fibrosis and may be used as a new therapeutic target(33).

When we performed a correlation analysis between HE4 and degree of fibrosis in the pre-dialysis group, we found a significant positive correlation between HE4 level and degree of fibrosis in the right kidney but not the

left kidney. This may be related to different micro vascular states of both kidneys. We investigated the correlations between renal fibrosis and laboratory parameters in pre-dialysis patients. We found a significant negative correlation between serum calcium level and degree of fibrosis.

Low serum calcium in the pre-dialysis group could be attributed to the reduction in vitamin D activation by the kidney due to reduction in kidney function, as vitamin D plays a pivotal role in calcium homeostasis. The vitamin D receptor activators (VDRAs) have a renal protective effect that hinders the development and progression of renal fibrosis as Vitamin D has an anti-inflammatory effect inhibiting the inflammatory process that aids in renal fibrosis progression. Moreover, vitamin D is instrumental in downregulating the renin-angiotensin system (RAS); a deficiency in vitamin D results in the activation of RAS, a pivotal mediator of renal damage. Recent research has illustrated that paricalcitol, a member of the VDRAs, can attenuate the elevation of renal TNF-α and the inflammatory infiltration provoked by uremia, thus impeding the progression of renal fibrosis (34). This provides a plausible explanation for our observed results.

In accordance with our results Wan et al. 2016 found that serum HE4 was inversely correlated with calcium levels ($r = -0.2821$; $P < 0.0001$) in CKD patients (32).

Also, we found a significant positive correlation between serum HE4 and serum phosphorus in the pre-dialysis group. In accordance with our results Wan et al. 2016 found that serum HE4 levels were positively correlated with phosphorus levels ($r = 0.2050$; $P < 0.0001$) (32).

The excess extracellular phosphate in renal cells lead to enhancement of the production of extracellular matrix (ECM) and pro-fibrotic molecules by fibroblasts which furtherly activate renal fibrosis and thus increase HE4 levels (35).

We found that the degree of renal fibrosis was higher in males than females in the dialysis group. Epidemiological studies indicate that CKD has a higher prevalence in females compared to males; however, the incidence of ESRD is greater in males. This disparity can be attributed to the continuous decline in renal blood flow in CKD patients, driven by vasoconstriction and capillary damage, which fosters renal fibrosis. In females, hormonal factors play a protective role by reducing the activity of the RAS, downregulating the angiotensin 1 receptor, and diminishing aldosterone production, thereby mitigating the progression of renal fibrosis (36).

Similar to our results, Zhao et al., (2020) found in their bi-directional Mendelian randomization study done on English CKD patients that CKD incidence was more prominent in males due to the effect of testosterone which might be an underlying cause of CKD and worse kidney function in men but not in women (37). We found a significant positive correlation between both HE4 level and incidence of diabetes in pre-dialysis patients. In accordance with our results, Zhang et al., (2019) found a strong association between increased serum HE4 and CKD (38). Elevated serum HE4 levels have been linked to an increased risk of developing CKD in patients with type 2 diabetes mellitus. Our research demonstrated that renal fibrosis, as detected by SWE, correlated with CKD stage and eGFR. This finding aligns with previous studies that reported a positive association between CKD and renal cortical stiffness, measured by both YM (39,40) and SWV (41). Additionally, studies employing super-sonic shear imaging (SSI), such as those by Samir et al. (2015) and Radulescu et al. (2019), indicated that SWE measurements in CKD patients were significantly elevated compared to healthy controls (42,43). Our study found no significant difference in renal cortical stiffness between pre-dialysis CKD patients and those with more advanced CKD, except when comparing the control group to the dialysis group. This observation is consistent with Leong et al. (2018) (40), who reported no significant differences among CKD stages 3, 4, and 5, and with Peride et al. (2016) (41), who similarly found no variation between CKD stages. In our analysis, the mean SWE values (in kPa) for pre-dialysis patients were 8.41 in the right kidney and 8.37 in the left kidney. For patients with CKD stages or on dialysis, the mean SWE values were 10.88 kPa in the right kidney and 10.83 kPa in the left kidney. In the control group, the mean SWE values were significantly lower, at 2.95 kPa in the right kidney and 2.71 kPa in the left kidney. Anwar & Tantawy (2021) reported mean YM values of 3.65±0.9, 4.5±1.2, 5.8±0.5, 5.3±1.1, and 6.6±0.9 for CKD stages 1, 2, 3, 4, and 5, respectively, with an overall mean value of 5.44±1.4 (39). Leong et al. (2018) documented mean YM values of 7.61±6.09, 11.61±6.88, 10.06±5.72, and 12.75±5.63 for CKD stages 2, 3, 4, and 5, respectively (40). In alignment with our findings, multiple studies employing SWE

techniques on human adult native kidneys have demonstrated a positive correlation between CKD and renal cortical stiffness (40,43,44). However, an exception was noted in the study by Danse et al. (2017), which found no such correlation (45).

Several studies have indicated that kidney SWE values are significantly elevated in patients with CKD compared to normal controls, highlighting increased renal stiffness attributable to chronic disease (46,47). Contrarily, other researchers have reported significantly lower renal stiffness in CKD patients (48,49). Despite observations in some studies that kidney SWE values decrease alongside the reduction in eGFR, elastography has not been successful in differentiating between the various stages of CKD, as no significant differences in shear wave speed (SWS) were detected across the different CKD stages (50,51). A meta-analysis encompassing seven studies, with a combined total of 639 CKD patients and 640 normal controls, demonstrated that kidney SWS is reduced in CKD patients. Furthermore, the analysis revealed a progressive decline in kidney SWS correlating with the advancement of CKD, as evidenced by the decreasing GFR (51). We performed ROC curve to assess the diagnostic performance of HE4 as biomarker to discriminate between pre-dialysis and dialysis groups and we found that at cutoff value of 8300 pmol/l HE4 could discriminate between pre-dialysis group (CKD stage 2-3) and dialysis group (CKD stage 4-5) and the degree of fibrosis with AUC= 0.884, sensitivity of 78.1% and specificity of 90%, $p < 0.001$.

In accordance with our results Song et al., 2023 (29) found that serum HE4 levels increased progressively with the progression of CKD stages, as well as the optimal cutoff value of serum HE4 351.5 pmol/L.

The analysis yielded an area under the curve (AUC) of 0.860 (95% confidence interval [CI]: 0.808–0.913; $p < 0.001$), demonstrating a sensitivity of 100% and a specificity of 66.5%.

In conclusion, combining the renal elastography and serum HE4 level measurement could serve as a diagnostic non-invasive panel for renal fibrosis instead of renal biopsy, and HE4 could be used as a biomarker for detection of CKD severity.

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