Sulaimani Journal for Pure and Applied Sciences Vol. 28 Issue 1 (2026) 43-59.

Journal homepage https://sjpas.univsul.edu.iq
Sulaimani Journal for Pure and Applied Sciences
ISSN:1812-4100

Original Article

Emerging Roles of Angiotensin-Converting Enzyme 2(ACE2)/Angiotensin-(1-9)[Ang-(1-9)] Axis in Hypertension Related Kidney Disease

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ARTICLE INFO

Article History:
Received: 19/12/2025
Revised: 03/03/2026
Accepted: 16/03/2026
Published online: 25/6/2026

Key Words:
ACE
Ang-(1-9)
ACE2
Hypertension
Kidney disease
RAS system

ABSTRACT

The Renin-Angiotensin System (RAAS) is a crucial regulator of blood pressure and kidney function. This study aimed to elucidate the role of key components of this system by evaluating their circulating concentrations and tissue expression patterns using enzyme-linked immunosorbent assay and quantitative polymerase chain reaction, with samples recruited from Shar and Ferkary Hospitals. A total of 90 blood samples were collected from three groups: controls, participants with kidney disease without hypertension (non-HTN-CKD), and participants with hypertensive kidney disease (HTN-CKD). In addition, 23 kidney biopsies were collected from participants at Shar Hospital; 13 of the participants had kidney disease associated with hypertension, whereas the remaining 10 had kidney disease without hypertension. Circulating Ang-(1-9) levels were markedly increased in patients with HTN-CKD (988.5±143.4) compared with those in the control group (716.3±288.0; P-value < 0.0001). In contrast, Angiotensin Converting Enzyme (ACE) concentrations were significantly lower in HTN-CKD patients (49.38±10.80) compared to the control group (70.74±21.73; p < 0.0001). While circulating ACE2 concentrations (mean ± SD) were as follows: HTN-CKD, 1.859±0.4646; non-HTN-CKD, 1.683±0.3397; control, 1.956±0.5467. In conclusion, these findings suggest that ACE2 plays a limited role in hypertensive kidney disease. In contrast, elevated Ang-(1-9) levels appear to be significantly associated with hypertension and kidney disease. In conclusion, further research with a larger sample size and investigation of additional RAAS components and related pathways is needed to better understand the role of this pathway.

1. Introduction

Hypertension remains one of the leading causes of chronic kidney disease (CKD) worldwide (He et al., 2025). Historically, Angiotensin II (Ang II) was considered the primary effector peptide of the Renin-Angiotensin System (RAS) (Stanek et al., 2025). The RAS pathway consists of a series of enzymatic reactions initiated by the secretion of renin (Sansoè &Aragno, 2023). The production of renin begins when prorenin is cleaved into renin upon activation of juxtaglomerular (JG) cells (Kulkarni et al., 2022; Shukla et al., 2024; Bayraktutan, 2025). Renin then cleaves angiotensinogen to form Angiotensin I (Ang I) (Silva et al., 2020; Norambuena-Soto et al., 2022), which is subsequently converted into Ang II by Angiotensin-Converting Enzyme (ACE) (Aimo et al., 2021). Ang II binds to its receptor, AT1R (Angiotensin II type 1 receptor), to mediate physiological responses,

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including vasoconstriction, increased water intake and Na retention, tissue remodelling, oxidative stress, and inflammation(Cohen et al., 2020). This series of actions is named the "classical" RAS (Ma et al., 2023; Sanad et al., 2023; Elgazzaz et al., 2024; Lang et al., 2025).

Conversely, Angiotensin-(1-7) [Ang-(1-7)] is the counter-regulatory arm of RAS, by binding to its receptor named Mas-R (Sarkar et al., 2024), works the same as Ang II through binding to AT2-R (Angiotensin II type 2 receptor), both work against the classical RAS (Karimi et al., 2024) causing vasodilation (Wang et al., 2023). Ang-(1-7) is also a ligand for Mas-related G-coupled receptor type D (Mrg-D) receptor (Sansoè &Aragno, 2023), by binding to Mrg-D, gives the same physiological response as Mas-R (Rajapaksha et al., 2021). Ang-(1-7) is produced by the action of Angiotensin Converting Enzyme 2 (ACE2) (Aimo et al., 2021; Turner &Nalivaeva, 2022). ACE2 converts Ang II to Ang-(1-7) and Ang I to a peptide known as Angiotensin-(1-9) [Ang-(1-9)] (Sotomayor-Flores et al., 2020). The Ang-(1-9) can also be cleaved to form Ang-(1-7) in the same way, but by ACE, Neprilysin, or Cathepsin A activity (Paz Ocaranza et al., 2020; Barzegar et al., 2021; Kunvariya et al., 2023). The ACE2 gene is expressed in many tissues, especially in the kidney, heart, and testes. (Shirbhate et al., 2021), in the form of a membrane-bound protein and in the soluble form (Hu et al., 2021; Liu et al., 2021; Wiese et al., 2021; Magazine et al., 2023). The soluble form of ACE2 is generated by the action of disintegrin and metalloproteinase 17 (ADAM17) (Stanek et al., 2025), an enzyme that is primarily activated after AT1R activation (Marquez et al., 2021; Kanugula et al., 2023).

The ACE2/Ang-(1-9) axis thought to be inactive axis since the Ang-(1-9) directly convert to Ang-(1-7)(Ocaranza &Jalil, 2012; Sotomayor-Flores et al., 2020). However, the specific role of Ang-(1-9) remains under investigation. This peptide acts mainly through AT2-R (Ocaranza &Jalil, 2012). Upon binding to this receptor, a series of reactions will be activated, including the nitric oxide synthase pathway (Zamai, 2020), vasodilation, and natriuresis, finally leading to low blood pressure (Kanugula et al., 2023).

This study aims to investigate the relationship between the ACE2/Ang-(1-9) pathway and kidney disease in patients with hypertension. ACE2 expression and activity were evaluated in patients with hypertensive CKD compared to those with hypertension but preserved kidney function. This approach may provide insight into whether a reduction in ACE2 expression is related to kidney injury in patients with hypertension. In addition, alterations in ACE2 or Ang-(1-9) levels correlate with the severity of kidney injury, thereby providing new insights into the management of hypertensive kidney disease.

2. Methodology and materials

2.1. Study design and ethical approval

This study started on the 21st of November of 2024 and is based on 90 blood samples from 90 participants recruited from two medical centres: Shar Hospital and Ferkary Hospital. In addition, 23 kidney biopsies were collected from patients at Shar Hospital. Ethical approval was granted by the institutional ethical committee in the College of Medicine, University of Sulaimani, Sulaimani City, under number 353, meeting number 25, and written informed consent was obtained before the sample collection.

2.2. Study group and criteria

Volunteers were categorized into three groups based on the results of the clinical examination and laboratory test results. To participate in the study before recruitment into each of the groups below, the following check-ups were conducted: Blood Pressure check (Omron corporation, Japan); Blood Sugar check (COBAS C111 and COBAS E411 analyser, Roche, Germany); and Clinical check-up involving Urea and Creatine (COBAS C111 and COBAS E411 analyser, Roche, Germany) tests and other biochemical parameters relevant to the study.

Control group (n=23): Participants with no known history of hypertension and chronic renal ailments.

Non-Hypertensive Chronic Kidney Diseases (non-HTN-CKD) group (n=31): This group comprises participants with chronic kidney disease who are not hypertensive. Participants were recruited from the Shar and Ferkary Hospitals. They were included because of their clinical presentations and the diagnostic workup performed. Their tests included Serum Creatinine, Urea, Sugar, Electrolytes, and Viral Studies.

Hypertensive Chronic Kidney Disease (HTN-CKD) group (n=36): The HTN-CKD group underwent the same diagnostic and treatment process as the non-HTN-CKD group. Nonetheless, all the patients belonging to the HTN-CKD group have a confirmed medical history of hypertension. A significant proportion of the patients in the HTN-CKD group, alongside those in the non-HTN-CKD group, have stage 3, 4, and 5 chronic kidney disease, based on their eGFR values, calculated via the CKD-EPI equation (2021, race-free), the race 2020 is an equation that uses the Creatine level, age, and the gender of the individual to extract the eGFR this equation does not require the race correction as developed by the National Kidney Foundation and the American Society of Nephrology(Gansevoort et al., 2023).

Certain factors are deemed exclusion criteria, which have been written below because these factors also impact the value of the variables being intervened upon by the team to ensure the outcomes are a result of the renal issue and no other factors:

- Age above 80 years and below 20 years.
- With renal cyst, especially polycystic
- Cardiovascular disease
- Infection with viruses

2.3. Tissue samples

Renal tissue biopsies were performed in collaboration with the nephrologist. A total of 23 samples were collected, and histopathological examination was performed on each biopsy to assess tissue morphology prior to further molecular analysis. Histopathological examination was performed independently by the hospital histopathologist as part of the routine clinical evaluation and wasn't part of the present study. Due to the limited size of the biopsy specimens, the available tissue was allocated exclusively for molecular analysis. The histopathology report was reviewed only to confirm the clinical diagnosis. Samples inconsistent with the diagnosis of kidney disease were excluded from the study. The clinical diagnosis and laboratory results that formed the basis for the sample groups are as follows: Hypertensive Kidney Disease (HTN-CKD) group, the test group comprising 10 native kidney biopsies and 3 transplanted kidney biopsies. All patients had confirmed hypertension and an unexplained decline in kidney function at the time of biopsy by the histopathologist. Non-Hypertensive Kidney Disease (non-HTN-CKD) group (n=10): Six of them were native kidney biopsies, and four were transplanted biopsies.

Immediately after collection, all biopsy samples were preserved in RNAlater and stored at -80 °C until use.

2.4. Blood collection and physiological measurements.

Whole blood (5ml) was obtained using a gel tube. The blood samples obtained were then centrifuged at 3000rpm for 10 minutes to obtain the serum. The serum was then further extracted and placed in sterile tubes. Immediately, the serum was stored in a freezer at -80°C. The serum samples were analysed for three biomarkers, namely Ang-(1-9), ACE, and ACE2, using an ELISA kit obtained from BT-LAB, China. All experimental protocols were carried out as per the instructions provided in the kit.

The absorbance in each well was determined at 450nm with a Gene5 microplate reader (BioTek, USA). For each set of samples, a standard curve was created using each assay to ensure that the sample concentrations were within the kit's linear quantification range.

2.5. Quantitative Real-Time PCR (qPCR)

Renal tissue biopsies were first homogenized under liquid nitrogen using the mortar and pestle to ensure complete tissue distribution. Total RNA was extracted from the homogenized tissue using the NZYtech RNA extraction kit (NZYtech, Germany), following the manufacture instruction, with slight modifications to step 1 and 2 of the original protocol, RNA extraction was initiated by adding 50µl of Qiazol reagent, followed by the addition of the chloroform to enhance phase separation and improve RNA yield, the remaining steps of the protocol were carried out as stated in the protocol. The purity and concentration of the extracted RNA were measured using a Nanodrop spectrophotometer (Thermo Fisher Scientific, USA). RNA was considered pure and acceptable for downstream applications only when the A260/A280 ratio was between 1.9 and 2.1.

From the RNA samples prepared and qualified, cDNA was synthesized using the NZYtech cDNA synthesis kit, following the provided instructions. The resulting cDNA samples were stored on ice and immediately used for qPCR.

Quantitative PCR was performed using the NZYtech SYBR Green qPCR kit to evaluate the gene expression of ACE1 and ACE2, and the analysis was conducted on the CFX96 deep-well real-time PCR detection system (Bio-Rad, Hercules, CA, USA). Gene expression levels were quantified using the ΔCt method. A comparison was conducted between two experimental groups: patients with non-HTN-CKD and those with HTN-CKD. All samples were run in duplicate to ensure reproducibility, and results were normalized to GAPDH and ACTB, which served as endogenous reference genes.

Relative gene expression was calculated using the 2^(-ΔΔCt) method, where ΔΔCt represents the changes in ACE and ACE2 between the HTN-CKD and non-HTN-CKD groups. The delta Ct (ΔCt) values represent the differences between the Ct (cycle threshold) of the target gene and the Ct of the reference (housekeeping) gene within the sample. The following formula was used for gene expression. This formula converts the logarithmic Ct differences into linear fold change, assuming PCR efficiency is close to %100. The fold changes between groups were statistically analysed using an independent t-test.

- ΔCt =C*t experimental*-Ct *control*
- ΔΔCt = value of sample (control or test)-average ΔCt of control
- Fold change=$2^{-\Delta\Delta C}$

2.6. Statistical analysis

The data analysis was performed using GraphPad Prism version 9.5.1. The normality of the data distribution was checked by the Shapiro-Wilk test. Comparisons among groups were performed using one-way ANOVA with Tukey's post hoc test or independent samples t-test for data showing normal distribution, while the Mann-Whitney or Kruskal-Wallis test was applied when comparing more than two groups if the data did not exhibit a normal distribution. Pearson's correlation for parametric data and Spearman's correlation for nonparametric data were calculated to assess continuous variables. ROC curve analysis was conducted for serum biomarkers to evaluate their diagnostic value, and AUC, sensitivity, specificity, and Youden index were reported. All data were presented as mean±standard deviation (SD), and a p-value ≤0.05 was considered statistically significant. All probabilities were 2-tailed.

3. Results

The biochemical tests performed for each group are summarized in Table 2 as mean ± standard deviation. Those patients with low Creatinine levels had other findings noted by the nephrologist, including proteinuria, abnormal ultrasound findings, edema, albuminuria, or abnormal urinalysis results. Most of the HTN-CKD patients had kidney failure, and they were on dialysis.

Table 1: The primers used in the analysis were custom-designed as follows:

Gene name	Forward primers	Reverse primer
ACE1	5-GGTGGTGTGGAACGAGTATG-3	5-CAGGGTGTGGTTGGCTATTT-3
ACE2	5-TGCTTGGTGATATGTGGGGT-3	5-TTTCCTGGGTCCGTTAGCAT-3
GAPDH	5-TCACCAGGGCTGCTTTTAAC-3	5-ATCGCCCCACTTGATTTTGG-3
ACTB	5-CACTCTTCCAGCCTTCCTTC-3	5-GTACAGGTCTTTGCGGATGT-3

Table 2: Demographic and biochemical characteristics of the study groups of blood samples.

Biochemical test	Control(C)	non-HTN-CKD groups	HTN-CKD groups	P-Value 1	P-Value 2	P-Value 3
Sex, M: F	7:16	9:22	14:22	-	-	-
Age	39.0±14.89	45.17±15.82	54.91±15.82	0.51	0.0007	0.04
ACE1 users	0%0	36%100	20%65	-	-	-
Creatinine (mg/dl)	0.76±0.118	2.503±2.891	6.489±4.227	0.02	<0.0001	<0.0001
Urea (mg/dl)	26.17±2.992	70.83±61.96	117.3±54.59	<0.0001	<0.0001	0.003
eGFR (ml/min/1.73m)	108.8±14.83	71.48±44.77	17.53±18.17	0.02	<0.0001	<0.0001
Na (mmol/L)	138.1±3.423	140.1±4.303	144.3±27.79	0.53	>0.99	>0.99
K (mmol/L)	4.153±0.332	4.333±0.5123	4.400±0.7565	0.55	0.40	>0.99
Blood sugar (mg/dL)	104.6±25.00	131.3±41.36	159.8±78.21	0.03	0.001	>0.99

*eGFR=estimated glomerular filtration rate.
*The P-value presented in each row follow this order: The first p-value represent the comparison between the control and non-HTN-CKD group; The second represents the comparison between control and HTN-CKD groups; and the third one represents the comparison between the non-HTN-CKD and HTN-CKD groups.

3.1. ELISA-based biomarker analysis

The expression pattern of Ang-(1-9) increased significantly in the studied group, with the highest values observed in patients with HTN-CKD. A significant increase in the non-HTN-CKD group compared with the control group was also observed; however, this did not reach statistical significance. On the other hand, the comparison within the HTN-CKD group revealed statistically significant differences, suggesting an association between high levels of Ang-(1-9) and hypertensive pathology in kidney disease.

The ROC curve analysis was done to assess the diagnostic potential of Ang-(1-9) for distinguishing among groups. Ang-(1-9) had an AUC of 0.8088 in discriminating HTN-CKD from control subjects (95% confidence interval of 0.676-0.905, p<0.0001) with a sensitivity of 82.3% and specificity of 66.67% at the optimal cut-off value of 846.014. These findings illustrate not only that Ang-(1-9) levels differ significantly among the studied groups, but also that Ang-(1-9) has strong discriminatory power for identifying patients with HTN-CKD, suggesting it could serve as a biomarker. For non-HTN-CKD, Ang-(1-9) had an AUC of 0.562 compared to controls (95% confidence interval of 0.406-0.709, p=0.5); despite a high sensitivity of 92.59%, the specificity was %27.78, indicating that though the test identified most of the true positive cases, it failed to

exclude the negatives. Overall, an AUC of 0.5 indicates that the biomarker does no better than chance at distinguishing between the two conditions, and its diagnostic utility in this context is limited despite its high sensitivity. These results are summarized in Figure 1, which provides the detailed descriptive statistics and adjusted p-values.

Unlike the Ang-(1-9) values, ACE2 gene expression did not differ significantly between groups in this study. Lack of differences could indicate that there isn't a strong relationship between gene expression and kidney disease in this group. Statistical analysis confirmed the relative stability of ACE2 levels among all groups. Although the ACE2 level didn't reveal differences among groups, the ROC analysis showed that ACE2 still has clinical value in a specific comparison. In distinguishing non-HTN-CKD from the control, ACE2 yielded an AUC of 0.672, with a borderline significant p-value of 0.050, at a cutoff of 2.046, with the 95% CI 0.516 to 0.804 (sensitivity 92%- specificity 50%), indicating a relatively strong ability to detect true positives and moderate discriminative accuracy. In contrast, the HTN-CKD AUC was 0.560 (p-value > 0.05; 95% CI, 0.412 to 0.700), indicating a limited role for ACE2 in hypertensive kidney disease, with a specificity of 27% and sensitivity of 90% at a cut-off value of 2.205.

Taken together, these findings imply that although overall differences in ACE2 level were not statistically significant, the ROC value suggests that ACE2 has clinical relevance in the non-HTN-CKD group, reflecting a possible pathophysiological specificity to non-hypertensive kidney diseases. Detailed data, including mean value, standard deviation, p-values, and ROC analysis, are presented in Figure 2.

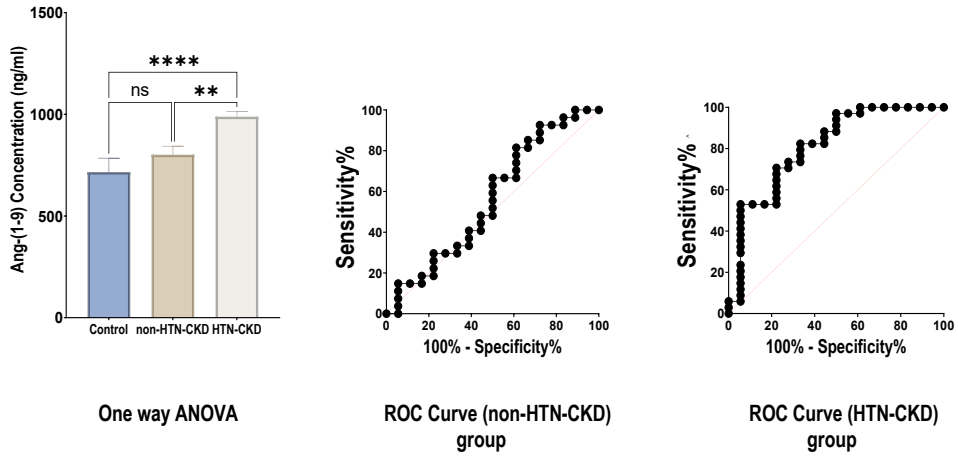

Figure 1. The graphical image shows the comparison of the Ang-(1-9) biomarker between the HTN-CKD, non-HTN-CKD, and control. The HTN-CKD level is higher in the HTN-CKD group than in the control group (988.5±143.4 vs. 716.3±288.0), with a p value <0.0001. Also, HTN-CKD compared to non-HTN-CKD has higher Ang-(1-9), with 988.5±143.4 vs. 801.7±214.3, p=0.0026. The non-HTN-CKD group also shows a higher Ang-(1-9) than the control group, with 801.7±214.3 vs. 716.3±288.0, but the difference is not statistically significant (p> 0.05). In the ROC curve, notice that the area under the curve indicates that the Ang-(1-9) has an important role in indicating the HTN-CKD more than the non-HTN-CKD, which shows a non-significant result with a low AUC, which is 0.5.

Figure 3 below shows the variations in the performance of ACE1 biomarkers among the study groups. A major finding is that ACE1 levels were highest in the control group and lowest in HTN-CKD patients. The trend indicated a significant decrease in ACE1 levels as the groups changed from the control to the HTN-CKD patients. The AUC of the ACE1 values for the HTN-CKD and non-HTN-CKD groups is 0.784 and 0.684, respectively. The AUC of the HTN-CKD indicates that ACE1 has excellent diagnostic capability (p-value = 0.002), with high specificity (66.67%) and sensitivity (88.67%) (CI

0.642 to 0.890), and an optimal cut-off of 64.736. In contrast, the non-HTN-CKD group had only a fairly good AUC of 0.684 (95% CI: 0.490-0.784), but again, this was not statistically significant (p=0.09). The sensitivity and specificity were 62.96% and 66.67%, respectively, at the optimal cut-off of 62.934. These results indicate that ACE1 diagnostic capability is excellent in the HTN-CKD groups compared with the non-HTN-CKD groups.

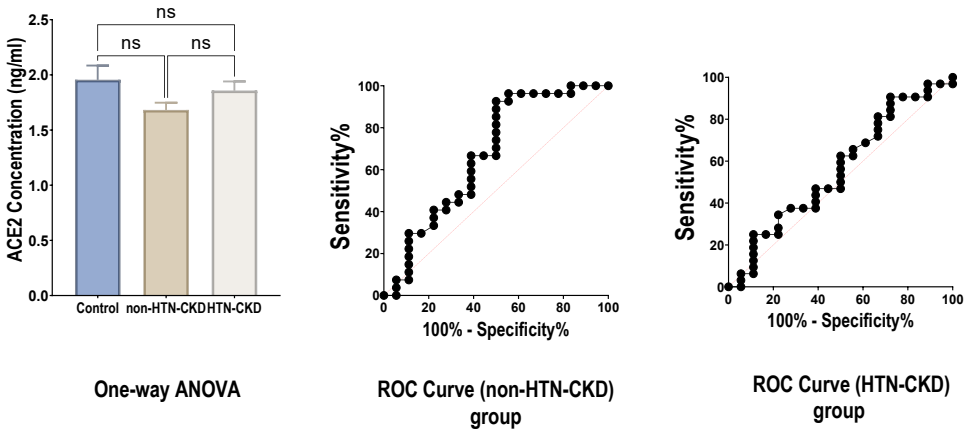

Figure 2: The ACE2 level across the study group revealed no meaningful variation between the cohorts. Despite apparent differences in Ang-(1-9) levels, ACE2 levels remained relatively unchanged, with no statistically significant differences between groups (p-values> 0.05). The mean and standard deviation of the groups are as follows: HTN-CKD: 1.859±0.4646, non-HTN-CKD: 1.683±0.3397, Control: 1.956±0.5467. The diagnostic performance of ACE2 was evaluated in both HTN-CKD and non-HTN-CKD groups using ROC curves.

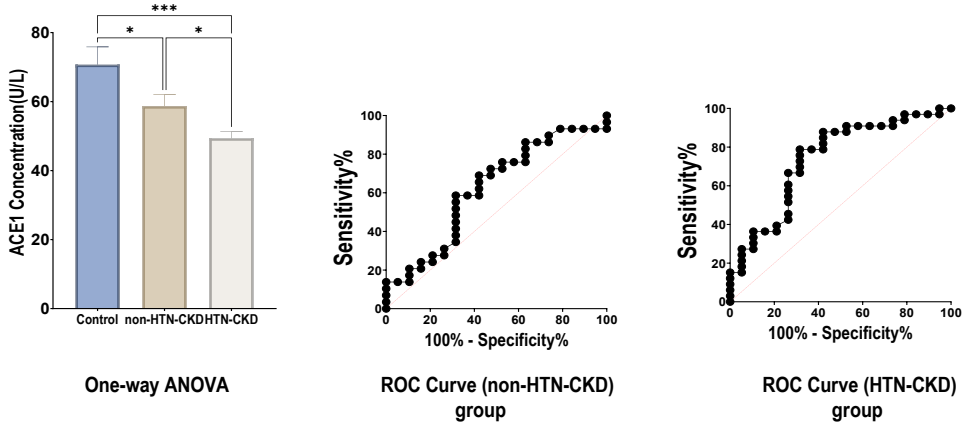

Figure 3: The graph shows the ACE1 levels in the HTN-CKD, non-HTN-CKD, and control groups. The highest bar in the bar graph is in the control group, indicating a higher ACE level (mean±standard deviation: 70.74±21.73), compared with the HTN-CKD and non-HTN-CKD groups (49.38±10.80 and 58.71±17.37, respectively). The same goes for non-HTN-CKD, which exhibited a higher mean±standard deviation of 58.71±17.37 compared to HTN-CKD 49.38±10.80, with p-values <0.05. The AUC analysis revealed that the ACE has strong diagnostic performance for HTN-CKD and is statistically reliable, whereas it shows moderate performance for non-HTN-CKD and cannot be relied on, as the p-value is greater than 0.05.

3.2. Correlation between the ELISA biomarkers

The correlation analysis among the various biomarkers in each group is shown in Figure 4. In non-HTN-CKD, a significant negative correlation was found between Ang-(1-9) and ACE2 levels (r=-0.4020, p-value=0.0377), suggesting that higher Ang-(1-9) levels were associated with lower ACE2 levels. In the control group, a positive relationship between ACE1 and ACE2 was observed (r = 0.6708; p-value = 0.0023), indicating that ACE1 and ACE2 are co-expressed at high levels in a healthy state. By contrast, the correlation patterns among the other

biomarkers were relatively weak and didn't reach statistical significance ($p > 0.05$). Therefore, these associations were considered biologically negligible and were not emphasized.

3.3. qPCR analysis of gene expression

When ACE1 expression was compared between groups, the non-HTN-CKD samples tended to show higher values than the HTN-CKD group, with a mean of 5.46±2.50. Although this difference suggested a trend toward increased ACE1 in the non-HTN-CKD group. For ACE2, the pattern was reversed. Here, the HTN-CKD group displayed a markedly higher average expression, 64.99±14.7, compared with the non-HTN-CKD group. Yet, as with ACE1, this difference did not reach statistical significance. Overall, while the expression points to opposite trends for ACE1 and ACE2 between the two groups, the data indicate that neither difference is statistically robust. These patterns are shown in Figure 5, where the spread of data within each group is clear from the box-and-whisker plots.

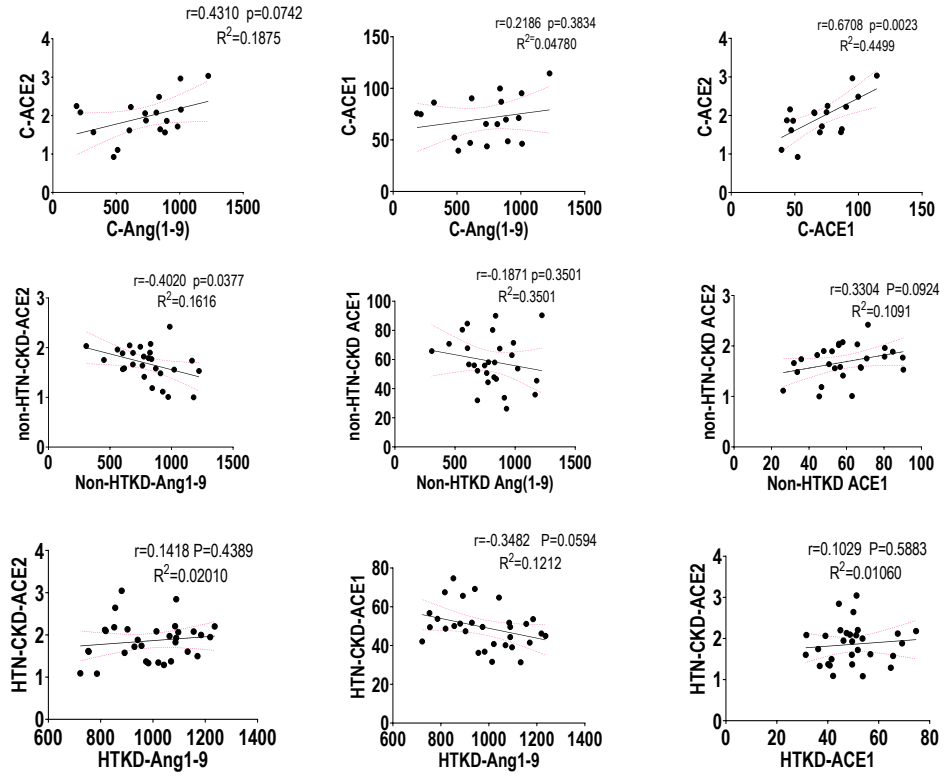

Figure 4: The correlation analysis between groups for each parameter is shown in detail. In the non-HTN-CKD, the only notable association observed was a negative correlation between Ang-(1-9) and ACE2. In contrast, the control group exhibited a strong positive correlation between ACE1 and ACE2. In the HTN-CKD group, Ang-(1-9) and ACE1 showed a weak negative correlation (p=0.059), suggesting a trend toward significance. Similarly, a weak positive correlation between ACE1 and ACE2 was observed in the non-HTN-CKD group (p=0.09), although it didn't reach statistical significance.

A summary of the relation between fibrosis and the expression level of both ACE1 and ACE2 is presented in Table 3. From a statistical viewpoint, no significant relations were found between fibrosis and expression of either gene. Similarly, no meaningful correlations were observed between either gene and renal function markers, including serum, creatinine, and eGFR. These findings indicate that, within the limits of the present study, neither fibrosis nor the biochemical tests studied are directly related to the expression of the ACE1 and ACE2.

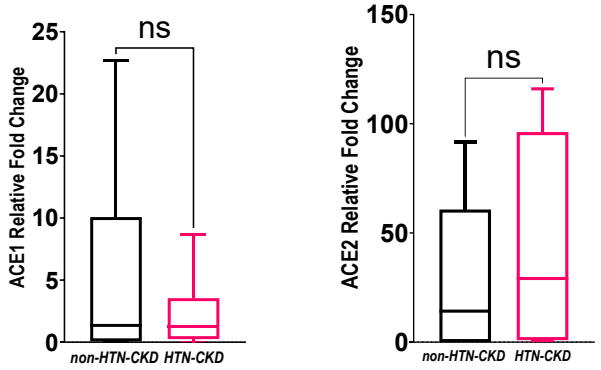

Figure 5: The result of the gene expression of both ACE1 and ACE2 in the kidney biopsy is shown; neither gene showed a statistically significant difference. Notice that in the ACE1 gene, the P value is 0.1769, with a high peak in the non-HTN-CKD group, while in the ACE2 gene, the P value is 0.3665, with the lowest peak in the non-HTN-CKD group.

Table 3: The correlation value of the fibrosis, biochemical parameters, and expression levels of ACE1 and ACE2.

Parameter	non HTN-CKD		HTN-CKD	
	ACE1	**ACE2**	**ACE1**	**ACE2**
Tubulointerstitial	r=0.288	r=0.026	r=-0.154	r=0.096
fibrosis	p=0.416	p=0.954	p=0.611	p=0.798
Creatine	r=-0.32	r=0.200	r=-0.329	r=0.443
	p=0.360	p=0.603	p=0.293	p=0.233
eGFR	r=0.561	r=-0.083	r=0.3357	r=-0.443
	p=0.096	p=0.833	p=0.286	p=0.233

*Significant at p<0.05.

4. Discussion

This study aimed to clarify the role of the Ang-(1-9) and ACE2 in hypertension-associated kidney disease. Both ACE2 and Ang-(1-9) belong to the antihypertensive arm of the RAS system; they act by counteracting the effect of Ang II and limiting ACE1 activity (Ocaranza &Jalil, 2012; Gonzalez et al., 2018; Sotomayor-Flores et al., 2020).

4.1. Biochemical tests

The biochemical data revealed significant alterations in several parameters among HTN-CKD compared with both non-HTN-CKD and control groups. Serum creatinine and urea levels were markedly elevated in HTN-CKD patients (p<0.001), reflecting reduced renal clearance and impaired kidney function. Conversely, eGFR values were significantly decreased in these patients (p<0.001), further confirming the decline in renal filtration efficiency associated with disease progression(Radeef et al., 2024). Sodium and Potassium concentrations showed no statistically significant differences between the groups(p>0.05), suggesting that electrolyte balance was relatively well maintained, possibly due to dietary control or medical management. Blood glucose levels were slightly higher in HTN-CKD patients than in controls (p=0.033), suggesting impaired glucose metabolism or the coexistence of metabolic disturbances commonly observed in CKD. In addition, a higher proportion of ACEI users was observed in the non-HTN-CKD and HTN-CKD groups compared with controls, consistent with the therapeutic use of ACEI inhibitors in hypertensive and nephropathic conditions. The observed increase in age and male predominance in CKD groups align with previous reports indicating that age is a significant risk factor influencing CKD prevalence and progression. (Carbayo et al., 2024).

4.2. ACE2 Biomarker and expression

Measuring circulating levels of ACE1, ACE2, and Ang-(1-9), as well as ACE1 and ACE2 expression, revealed that ACE2 did not play a significant role in CKD. Neither in circulation nor within the kidney did ACE2 show meaningful involvement. It is important to highlight that ACE2 has received increasing attention in recent years, particularly during the COVID-19 pandemic, when it was considered a potential therapeutic target. Many papers have proposed ACE2 as a candidate for treating viral infections and other diseases, including kidney diseases. This strong interest encouraged further investigation into its role in CKD.

These findings are consistent with work by (Jiang et al., 2020) and (Choi et al., 2020), who both reported no meaningful role of ACE2 in hypertension. The work by Choi

et al., which administered ACE2 to mice and found no improvement in renal function or even a reduction in blood pressure, suggests that it may increase Urinary ACE2. Animal studies provide conflicting evidence, for example, a study by (Lely et al., 2004), worked on diabetic nephropathy, and they found that in diabetic nephropathy, the ACE2 expression was downregulated compared to the ACE1, or the one by (Dilauro et al., 2010) found that ACE2 levels were downregulated, especially in early CKD. (Liu et al., 2012) found that the ACE2 is fluctuating, as in some CKD, is unchanged, as in SHRSP rats, while in some other diabetic nephropathy the ACE2 level increases or decreases; such discrepancies may be explained by differences in age, pathophysiology, diet, and environment of the studied subject, as in a study by (Fang et al., 2013), done on mice, they found that the deletion of ACE2 in mice showed an age-dependent kidney disease(Hekmat et al., 2019)have proved that such factors may alter ACE2 expression. However, another investigation (Jiang et al., 2020) found no influence of these factors, reinforcing the idea that ACE2 has no active role in hypertension.

In this cohort, most patients were receiving the ACE1 inhibitors, raising the possibility that treatment might have influenced the results.(Ocaranza et al., 2006), observed that ACE1 inhibitors like enalapril increase the ACE2 expression. They also mention that not only ACE1 inhibitors but also losartan(ARBs, angiotensin II receptor blockers) increase both ACE2 activity and expression, not only the RAS inhibitor drugs, but also ROCK(Rho-associated coiled-coil containing kinase) inhibitor drugs like Fasuldi increase ACE2 level, as it has been proven again by (Ocaranza et al., 2011). While in this study, ACE1 inhibitors didn't appear to alter ACE2 expression, the same result as our study was also obtained by studies from (Tipnis et al., 2000; Tikellis et al., 2003; Lely et al., 2004). They all found that ACE2 activity and expression were unaffected when measured in experimental animals. Taken together, the results in this current study indicate that ACE2 has no significant effect on hypertension or kidney disease.

4.3. Ang-(1-9) biomarker

In this study, circulating Ang-(1-9) levels were significantly elevated in HTN-CKD compared with both non-HTN-CKD and control groups. This observation contrasts with many earlier reports, in which Ang-(1-9) was either found at negligible levels or considered unstable due to its rapid hydrolysis to Ang-(1-7). However, this study's data aligns with studies that have described an inverse relationship between Ang-(1-9) and ACE1 activity, suggesting that suppression of ACE1 activity may contribute to the preservation and accumulation of Ang-(1-9).

Ang-(1-9) is a relatively recently characterized peptide, and its therapeutic relevance within the RAS is increasingly recognized. Although much of the existing literature has emphasized its cardioprotective effects, particularly in attenuating cardiac remodelling and delaying the progression of hypertension and cardiovascular disease, our findings highlight its potential role in renal pathophysiology. The significant increase in Ang-(1-9) observed in our cohort suggests it may exert protective effects not only against hypertension but also in kidney injury, offering a dual therapeutic avenue.

The pharmacological background of patients must also be considered. The majority of enrolled subjects were receiving ACE1 inhibitors, and this therapeutic factor likely explains both the reduction in ACE1 activity and the concomitant rise in Ang-(1-9). As ACE1 inhibition prevents the hydrolysis of Ang-(1-9) into Ang-(1-7), the peptide accumulates and may act as an independent protective mediator. These findings are in accordance with (Mendoza-Torres et al., 2018), which demonstrate that ACE1 inhibition is associated with an increase in circulating Ang-(1-9) and a corresponding decrease in ACE1 activity same to the study by (Gonzalez et al., 2018)They proved that drugs like enalapril or receptor antagonist blockers increased Ang-1-9 in circulation by 14-fold, while decreasing or

suppressing ACE1 expression and activity. A research by (Ocaranza et al., 2011) worked on rats who had cardiac diseases. The level of Ang-1-9 at the beginning of the study was higher than in the severe cases, while ACE1 increased excessively compared to the control. This level reversed when the same rats were treated with the ACE1 inhibitor.

In (Wysocki et al., 2010) Research on rats that were injected with Ang-(1-7) did not show improvement in hypertension, while administration of Ang-(1-9) not only improved the hypertension but also increased ACE2 activity and reduced heart disease (Norambuena-Soto et al., 2020). Further research is still needed to understand the role of Ang-(1-9) and how to replace the kidney and hypertensive drugs with Ang-(1-9) since it has an important impact on hypertension.

As the levels of Ang-(1-9) depend on ACE2 levels, as noted in many studies, it is important to ask in this study whether ACE2 levels are affected and, if so, how the increase in Ang-(1-9) occurs. Although ACE2 is considered the principal enzyme responsible for converting Ang I to Ang-(1-9) and Ang-(1-7), evidence suggests that alternative enzymatic pathways are involved, given that the RAAS is a complex system. For instance, Cathepsin A and Carboxypeptidase have been reported to hydrolyse the Ang I into Ang(1-9)(Ocaranza &Jalil, 2012; Martyniak &Tomasik, 2022). In addition, Neprilysin, Prolyl-endopeptidase, and Thimet-Oligopeptidase can convert Ang I directly into Ang-(1-7)(Marquez et al., 2021). Furthermore, Prolylcarboxypeptidase (PRCP) and Prolyl endopeptidase (POP) can directly change Ang II to Ang-(1-7) (Marquez et al., 2021).

4.4. ACE1 activity and expression

In contrast, ACE1 expression in kidney biopsy samples didn't differ significantly between groups, while there was a significant difference in the circulation between the groups. This result diverges from much of the published evidence, which generally reports increases in ACE1 levels in kidney diseases, both local and systemic, particularly in diabetic nephropathy, as mentioned by (da Silva Reis et al., 2021). The most plausible explanation in that pharmacological inhibition of ACE1 not only suppresses enzymatic activity but may also alter expression dynamics, in a paper by (Shi et al., 2020) it has been mentioned that taking the ACEI every day causes a reduction in ACE1 level, similar to research done by (Chen et al., 2023) demonstrated that giving Captopril to the mouse caused a decrease in ACE1 activity. While these observations support the hypothesis that Ang-(1-9) may act as a counter-regulatory peptide within the RAS, further work is essential. Studies exploring the direct administration of Ang-(1-9) or in vitro investigations at the renal tissue level could help clarify its specific role in modulating hypertensive kidney diseases.

4.5. Correlation of ACE, ACE2, and Ang-(1-9)

Positive correlations between circulating Ang-(1–9) and ACE2/ACE1 in the pooled cohort likely reflect adaptive remodelling of the RAS in response to hypertensive stimuli. While direct quantification of Ang-(1–9) in humans remains limited, studies have shown that higher plasma levels of ACE2 are associated with improved renal outcomes in chronic kidney disease, underlining the protective role of ACE2 in counterbalancing angiotensin II–mediated effects(Ueno et al., 2024). In line with our correlations suggesting that increased Ang-(1–9), a product generated when ACE2 acts on substrate, may reflect an upregulated counter-regulatory axis that tries to neutralize deleterious ACE1/Ang II activity. The ability of ACE2 to shift the peptide balance toward vasodilatory and antifibrotic fragments mechanistically explains why ACE2 and Ang-(1–9) concentrations would rise in parallel with ACE1 activity under conditions of sustained RAS activation.

In stratifications by disease status, the negative association of Ang-(1–9) with ACE2 in the non-HTN-CKD group suggests maintenance of the homeostatic

balance inherent in the renin-angiotensin system. This implies that efficient processing of Ang I and II peptides reduces overexpression of the classical ACE1/Ang II axis. This is because animal studies indicate that the impairment of ACE2 exacerbates hypertensive renal injury, suggesting that the competent expression of ACE2 is pivotal in arresting the progression of nephropathy through its enzymatic activity on angiotensin peptides(Chen et al., 2023). This implies that high ACE2 expression levels observed in the non-HTN-CKD group contribute to optimal processing rates and reduced pathological expression.

By contrast, the lack of significant correlations in the HTN-CKD subgroup may represent a functional uncoupling of the protective RAS axis in the context of chronic disease. Translational evidence from clinical studies has shown that renal ACE2 levels are positively correlated with markers of renal health but are not dysregulated by hypertension per se; rather, the disturbances in local RAS homeostasis in disease are thought to reflect downstream effects of changes in gene expression rather than dysregulation in abundance of the transcripts themselves((Jiang et al., 2020). This functional uncoupling may be due to post-translational changes, reduced enzyme availability for transcription activation with increasing peptide concentrations in pathological conditions, and a greater prevalence of the ACE1 peptide. Overall, these data highlight the complex interactions that regulate RAS in hypertensive kidney disease and provide evidence that dynamic changes in Ang-(1-9) and ACE2 are sensitive markers of RAS dysregulation rather than quantitative markers of enzyme abundance.

4.6. Correlation of ACE and ACE2 with fibrosis

These differing patterns of correlation between ACE1 and ACE2, and between indicators of renal function and structure, in both non-HTN-CKD and HTN-CKD patients also underscore the specific characteristics of RAS in each condition. That the lack of significant correlation of systemic ACE2 levels to l fibrosis or functional severity in the absence of hypertensive stress in the setting of CKD makes systemic ACE2 potentially irrelevant to the process of initial renal structure impairment in the absence of hypertensive stress in CKD patients also correlates with the observational data on the protective relationship of plasma ACE2 levels to the rate of change of the rate of decline of the eGFR and albuminuria in patients with diabetes and renal impairment(Ueno et al., 2024).

In the HTN-CKD group, the stronger correlations between ACE2 and functional parameters, such as creatinine levels and reduced eGFR, reflect a progressively closer link between systemic ACE2 expression and reduced renal function in chronic hypertensive conditions. Differing from structural fibrosis, which is a cumulative index of irreversible biological tissue injury, functional parameters such as eGFR and creatinine values are sensitive to hemodynamic and metabolic alterations. Data suggest that plasma ACE2 levels progressively come under the influence of functional impairment with increasing disease severity, perhaps via hypertensive stress-induced alterations of RAS activity and ACE2 shedding. Though few human studies have explored the relationships between systemic ACE1/ACE2 expression levels and the index of fibrosis, the correlation between plasma ACE2 levels and functional indices such as reduced eGFR has established systemic ACE2 expression levels as a functional prognostic marker for CKD progression (Ueno et al., 2024). Weaker correlations for ACE1 might suggest that its predominant role in local Ang II production is unrelated to systemic enzyme expression levels.

Most importantly, the absence of any meaningful correlation between ACE1 or ACE2 and fibrosis in both groups strongly argues against the possibility that systemic RAS components accurately reflect histopathological fibrosis levels. Fibrosis is an end-stage phenomenon resulting from intrarenal mechanisms involving profibrotic pathways such as

TGF-β, which cannot be directly predicted from circulating enzyme levels. The present level of evidence, as revealed by tissue expressions, argues in Favor of intrarenal ACE2 expressions being linked with eGFR and age, but being variably related to inflammation as well as fibrosis gene transcripts, which clearly reflects a compartmentalized phenomenon of RAS regulation in the kidney, distinct from circulating levels (Maksimowski et al., 2020). These observations argue in Favor of circulating ACE2 being more linked with functional rather than structural changes, particularly in hypertensive chronic kidney disease.

Limitations of this study were: First, the availability of patient samples was limited, as nephropathology remains a developing field in Iraq, and ethical and cultural considerations make it nearly impossible to obtain control kidney biopsies in Kurdistan. Secondly, we did not quantify Ang-(1-9) at the level of local renal tissue; measuring intrarenal peptide concentrations could have provided additional mechanistic insights. Thirdly, this study was conducted at a single center, which may limit the external validity of the results. Finally, the relatively small sample size may limit the generalizability of the findings.

5. Conclusion and perspective

In summary, this study shows that Ang-(1-9) levels are elevated in the HTN-CKD groups, suggesting that this peptide has excellent therapeutic potential. The inverse relationship between ACE1 and Ang-(1-9) in the HTN-CKD groups was likely due to the patients' ACE1 inhibitor therapy. On the other hand, the limited role of the ACE2 in the HTN-CKD group based on the facts that the ACE2 showed no significant alteration in it expression and in circulation activity among groups in the studied cohort, this results cast doubts on the notion that ACE2 even has role in the hypertension renal diseases and highlight the need for more researches on Ang-(1-9) as a potential new target for treatment of hypertension-induced kidney disease. From our perspective, future studies should investigate whether pharmacological activation of the ACE2/Ang-(1-9) pathway can provide therapeutic benefits. Expanding the analysis to larger patient populations and experimental models could further clarify this mechanism.

Conflict of interest

The authors have no conflicts of interest to declare.

Fund

No sponsor or funder supported this study.

CRediT authorship contribution statement

Conceptualization, methodology, investigation, data curation, formal analysis, writing-original draft, **Sima A. Mohammed**; writing-review & editing, supervision, project administration, **Ridha H. Hussien**. Both authors have read and agreed to the published version of the manuscript.

Acknowledgments

The authors would like to express their sincere gratitude to the administration and medical staff of Shar and Ferkari Hospital for their cooperation and assistance during sample collection process. Great thanks to Dr. Bnar Sardar Saida, a nephrologist at Shar Hospital, who helped us collect the kidney biopsies.

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