Title: Circulating Angiotensin Converting Enzyme 2 activity in patients with chronic kidney disease without previous history of cardiovascular disease.

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Running Head: ACE2 in CKD patients.

Abstract

Background. Circulating Angiotensin Converting Enzyme 2 (ACE2) activity is increased in patients with cardiovascular (CV) disease, but there is little information about changes in ACE2 in chronic kidney disease (CKD) patients without history of cardiovascular disease. We examined circulating ACE2 activity in CKD patients stages 3-5 (CKD3-5) and dialysis (CKD5D) without any history of CV disease.

Methods. Circulating ACE2 activity was measured in human EDTA plasma samples from the NEFRONA study (n=2572): control group (CONT) (n=568), CKD3-5 (n=1458) and CKD5D (n=546). Different clinical and analytical variables such as gender, age; history of diabetes mellitus (DM), dyslipidemia, hypertension; glycemic, renal, lipid and anemia profiles; vitamin D analogues treatment and antihypertensive treatments (ACEi and ARBs) were analyzed. Circulating ACE2 and ACE activities were measured using modified fluorimetric assay for EDTA-plasma samples, where zinc chloride was added to recover enzymatic activity.

Results. In CKD3-5 and CKD5D circulating ACE2 activity was significantly decreased as compared to CONT, but no differences were found between CKD3-5 and CKD5 when performing paired case-control studies. By multivariate linear regression analysis male gender and advanced age were identified as independent predictors of ACE2 activity in all groups. Diabetes was identified as independent predictor of ACE2 activity in CKD3-5. Circulating ACE activity was significantly increased in CKD3-5 and CKD5D as compared to CONT, and in CKD5D as compared to CKD3-5. By multiple regression analysis, female gender and younger age were identified as independent predictors of ACE activity in CONT and CKD3-5. Diabetes was also identified as an independent predictor of ACE activity in CKD3-5 patients.

Conclusions. Circulating ACE2 and ACE activity can be measured in human EDTA plasma samples with zinc added to recover enzymatic activity. In a CKD population without previous history of CV disease, ACE2 activity from human EDTA plasma samples directly correlated with the classical CV risk factors namely older age, diabetes and male gender. Our data suggest that circulating ACE2 is altered in CKD patients at risk for CV event.

Key words: ACE2, renin angiotensin system, chronic kidney disease, cardiovascular disease, biomarkers, diabetes.

Short summary

Circulating ACE2 and ACE activity can be measured in human EDTA plasma samples with zinc added to recover enzymatic activity. In a CKD population without previous history of CV disease, ACE2 activity correlates with the classical CV risk factors namely older age, diabetes and male gender, suggesting that ACE2 could have therapeutic implications for CV disease in CKD patients.

Introduction

Patients with chronic kidney disease (CKD) have an increased cardiovascular (CV) risk that accounts for more than 50% of the overall mortality [1]. Previous studies have found an independent association between lower levels of the estimated glomerular filtration rate (GFR) and the risk of CV disease (death, CV events and hospitalization). This risk is evident at an estimated GFR<60mL/min/1.73m^2, and increases with an estimated GFR<45mL/min/1.73m^2 [2]. The mechanisms that contribute to the pathogenesis of CV disease in CKD are complex, and include both traditional and non-traditional CV risk factors [3]. Nonetheless, it is well known that enhanced activation of the renin-angiotensin system (RAS), among others, plays a major role in the progression of cardiac and renal injury [4].

Within the RAS, the Angiotensin-Converting Enzyme (ACE) [5] converts Angiotensin(Ang) I into the vasoconstrictor AngII, which mediates its effects predominantly through angiotensin type 1 receptor and is responsible for the pathophysiological effects of the RAS. AngII increases blood pressure and contributes to cardiac remodeling, fibrosis, inflammation, thrombosis and plaque rupture [6]. In 2000, ACE2, an enzyme that cleaves the C-terminal amino acid of AngII to generate the peptide Ang1-7 was identified [7]. Ang1-7 acts via the Mas receptor to counteract the adverse effects of AngII [8]. Previous experimental studies have reported that downregulation of ACE2 leads to age-dependent development of glomerular mesangial expansion, accelerated progression of glomerulosclerosis, tubular injury, macrophage infiltration and interstitial fibrosis [9-11].

ACE2 is an integral cell membrane protein that can undergo cleavage or shedding to release the catalytically active ectodomain into the circulation. Initial studies in a large cohort were able to detect circulating ACE2 activity in only 7.5% of the subjects and it was~100-fold lower than ACE [12]. Interestingly, subjects with detectable ACE2 were older than those without and had a higher prevalence of CV disease, diabetes and hypertension suggesting that ACE2 may be upregulated in subjects with CV disease to counteract the adverse effect of AngII. Subsequent studies demonstrated that circulating ACE2 activity could also be detected in healthy subjects [13] and that soluble ACE2 activity is increased in heart failure patients, acute myocardial infarction[14], and correlates with the severity of the heart disease [15]. Circulating ACE2 levels

are increased in patients with type 1 diabetes and vascular complications [16]. Our group also demonstrated that circulating ACE2 activity can be measured in kidney transplant patients (KT), suggesting that it may be used as a non-invasive marker to understand the role of RAS in KT [17]. Recently, Roberts et al. showed that plasma ACE2 activity was lower in patients undergoing hemodialysis than in pre-dialysis patients with CKD [18]. However, circulating ACE2 activity has not been studied in CKD patients without previous history of CV events. Therefore, the aim of this study was to determine the levels of circulating ACE2, ACE, and renin activities in CKD patients without any history of CV disease, and to determine the factors associated with circulating ACE2 and ACE activities in this population.

Subjects and Methods

Patients and Variables

2572 subjects from an observational and multicenter study (NEFRONA project), recruited from October 2009 to June 2011, were studied [19, 20]. Male and female patients without history of CV disease (angina pectoris, acute myocardial infarction, ischemic stroke, hemorrhagic stroke, abdominal aortic aneurysm and atherosclerosis), and ages ranged between 18 and 74 years old were included in the study. Exclusion criteria were pregnancy, VIH infection, any type of transplant or history of transplant, previous history of carotid artery disease, patients with active infections and/or hospitalized in the last month, and intercurrent illness that presumes absence of follow-up or survival expectation less than 1year.

Patients were classified into three groups according to their glomerular filtration rate (MDRD-4): 1458 non-dialysis patients with CKD stages 3-5 (CKD3-5, MDRD-4<60ml/min/1.73m^2); 546 dialysis(hemodialysis or peritoneal dialysis) patients (CKD5D); and 568 subjects with MDRD-4≥60ml/min/1.73m^2 were used as controls (CONT).

Clinical variables analyzed were gender, age, history of diabetes, hypertension, dyslipidemia, and smoking (active smokers over the last month). Angiotensin Converting Enzyme inhibitor (ACEi), Angiotensin Receptor Blockade (ARBs), diabetes medication (insulin and oral antidiabetic drugs), and vitamin D analogues treatments were recorded. Analytical variables

analyzed were: glycemic (blood glucose and glycosylated hemoglobin), lipid and anemia profiles. Presence of plaques was determined by ultrasound of the carotid arteries.

EDTA-anticoagulated plasma samples were collected from all patients and controls, centrifuged at 3000g and then frozen at –80°C until analysis. The protocol has been reviewed and approved by the ethical review board of each hospital, and each participant signed an informed consent document before being included into the study.

ACE2 Enzymatic Assay

The ACE2 fluorescent enzymatic assay protocol was performed as previously described by our group, using an ACE2-quenched fluorescent substrate (Mca-Ala-Pro-Lys(Dnp)-OH, BioMol; Enzo, Life Sciences)[14, 17, 21]. Plasma samples were collected into tubes containing ethylenediamine-tetraacetic acid (EDTA), a chelating agent of loosely bound metal ions such as $Zinc^{2+}$, which acts as a cofactor for carboxypeptidases. As EDTA inhibits ACE2 and ACE activity [22], zinc chloride($ZnCl_2$) was added to the plasma samples to avoid its bounding. Briefly, 2µl of plasma were incubated with buffer(100mM Tris-HCl, 600mM NaCl, 10µM $ZnCl_2$, pH 7.5) in the presence of protease inhibitors(100µM captopril, 5µM amastatin, 5µM bestatin and 10µM Z-Pro-prolinal) and in the presence of different concentrations of $ZnCl_2$ (0, 0.5, 1 and 3mM) (Figure 1A). Samples were incubated with 20µM of the quenched fluorescent substrate in reaction buffer (final volume 100µL) at 37ºC for 16hours. The optimal concentration of $ZnCl_2$ for the determination of ACE2 activity was 0.5mM. ACE2 activity was also calculated in heparin-plasma and serum samples from 21 subjects as previously described [14,17].We performed a standard curve of the rhACE2 adding increasing quantities of rhACE2 (0, 1, 2, 4, 8 and 16ng) (Figure 1C). In a set of experiments, serum and EDTA-plasma samples were incubated with human recombinant ACE2 (Calbiochem) in presence or absence of $ZnCl_2$. Experiments were carried out in duplicate and results were expressed as RFU/µL plasma/hour.

ACE Enzymatic Assay

The ACE fluorescent enzymatic assay was performed as previously described with modifications [23]. $ZnCl_2$ was tested to find the optimal concentration for reversing the effect of EDTA. Briefly, 0.83µL of plasma with different concentrations of $ZnCl_2$ (0, 7.81, 15.63 and

31.25mM) (Figure 1B) were incubated with 73µL of appropriate buffer (0.5M borate buffer and 5.45M N-hippuryl-His-Leu (HHL)) at 37ºC for 25minutes. Finally, the fluorescent adduct of the enzyme-catalyzed product L-histidyl-L-leucine was quantified. A concentration of 15.63mM was found to be optimal for the detection of ACE. ACE activity was also calculated in heparin-plasma and serum samples from 21 subjects as previously described [17]. Experiments were carried out in duplicate and results were expressed as RFU/µL plasma.

Statistical Analysis

Normality of the continuous variables was assessed by normal probability plots. Variables were expressed as mean±SE. Paired case-control studies were performed: CONT versus CKD3-5 (280 pairs), CONT versus CKD5D (188 pairs) and CKD3-5 versus CKD5D (360 pairs), with an equal distribution of gender, diabetes, hypertension, dyslipidemia, smoking habits, weight(± 5 kg) and age (± 3 years). Continuous variables were evaluated by the non-parametric Mann-Whitney test. Bivariate correlations were calculated by the Spearman's correlation coefficient. ANOVA was used among plasma ACE2 and ACE activity. The intraclass correlation coefficient (ICC) was used to determine the concordance of the assay between the different human samples. Multiple linear regression analyses, using the natural logarithmic transformation of plasma ACE2 and ACE activity, were carried out to identify independent predictors of enzymatic activity. *SPSS* version 18.0 for Windows was used for statistical calculations and R package version 3.0.2 was used for case-control studies. $P<0.05$ was considered statistically significant.

Results

Patient characteristics

In total, 2572 patients were included in the study. Circulating ACE2 and ACE activities were measured in 568 CONT subjects and 2004 CKD patients without previous history of cardiovascular disease, divided into those not requiring dialysis(CKD3-5) and those in dialysis(CKD5D). Characteristics of study subjects are shown in Table 1. CKD5D were younger and thinner than CKD3-5. CKD3-5 and CKD5D patients had higher prevalence of hypertension and dyslipidemia than CONT. NEFRONA is the first large study describing the actual prevalence of subclinical atheromatosis across different CKD stages. The baseline atherosclerosis parameters of the patients have been detailed by Arroyo D. et al. [24].

ACE2 and ACE activities in EDTA-plasma samples

We studied ACE and ACE2 activities in EDTA-plasma samples. For this purpose, ACE and ACE2 activities were measured in different conditions: EDTA-plasma samples, EDTA-plasma samples with added ZnCl2, serum samples and plasma samples collected on heparin from 21 subjects

We found a strong correlation with ICC (≥0.84) between the different studied samples (plasma on EDTA+ZnCl2, heparin and serum) for both ACE2 and ACE activities. In addition, we were able to recover ≥91% of ACE2 activity and ≥83% of ACE activity from EDTA-plasma when $ZnCl_2$ was added to the assays (Table 2).

In concordance with the non-detectable ACE2 activity observed in EDTA-plasma samples, when rhACE2 was added to the EDTA-plasma, ACE2 activity was not observed (Figure 1D). As expected, when $ZnCl_2$ was added to the EDTA-plasma with rhACE2, ACE2 activity was detected. These results, demonstrated that within EDTA-plasma neither endogenous or exogenous ACE2 activities were detected, however the addition of zinc to the reaction was able to recover the enzymatic activity.

ACE2 activity

Circulating ACE2 activity was significantly decreased in CKD3-5 as compared to CONT (45.4±1.12 versus 52.9±1.50, $p<0.001$) and in CKD5D as compared to CONT (38.5±1.62 versus

52.9±1.50, p<0.001). In addition, ACE2 was significantly decreased in CKD5D as compared to CKD3-5 (38.5±1.62 versus 45.4±1.12, p<0.001)(Figure 2A). However, when paired case-control studies were performed, no differences between CKD3-5 and CKD5D were found (p=0.27)(Figure 2B). Therefore, we analyzed all groups separately.

ACE2 activity was significantly increased in males as compared to females in all studied groups (p<0.001). Furthermore, ACE2 activity was also increased in patients with plaques as compared to those without plaques (p<0.001)(Table 3).

CONT and CKD3-5 with diabetes showed increased circulating ACE2 activity as compared to non-diabetic patients (p=0.003 and p<0.001). However, no differences were observed in dialysis patients (p=0.60). Hypertension was also associated with increased ACE2 activity in CONT (p<0.001)(Table 3). Patients with dyslipidemia showed increased levels of circulating ACE2 in CONT (p<0.001) and CKD5D (p=0.028), but no differences were found in CKD3-5 (p=0.53). Interestingly, circulating ACE2 activity was significantly increased in smoker as compared to non-smoker CKD3-5 (p=0.03)(Table 3).

We found a significant direct correlation between ACE2, age and glycosylated hemoglobin in both CONT (p<0.001) and CKD3-5 (p<0.05). In addition, a direct correlation between ACE2 and age was found in CKD5D (p=0.038).

Circulating ACE2 activity was significantly increased in CONT and CKD5D on ARBs therapy as compared to non-treated patients (p=0.002). Treatment with ACEi did not influence circulating ACE2 (Table 3). ACE2 activity was also increased in CONT (p=0.007) and CKD3-5 (p<0.001) under oral antidiabetic agents as compared to non-treated. In addition, insulin therapy increased ACE2 activity in CKD3-5 (p<0.001)(Table 3). Surprisingly, circulating ACE2 was decreased in CKD5D treated with cholecalciferol as compared to non-treated patients (p=0.027)(Table 3).

By multivariate linear regression analysis (Table 4), male gender and advanced age were identified as independent predictors of circulating ACE2 activity in all studied groups. Diabetes was also identified as independent predictor of ACE2 activity in CKD3-5. In addition, ARBs and cholecalciferol therapies were independent predictors of ACE2 in CKD5D.

ACE activity

Circulating ACE activity was significantly increased in CKD3-5 as compared to CONT (4181.65±58.37 versus 3809.13±71.96, p=0.035) and in CKD5D as compared to CONT (4454.48±87.10 versus 3809.13±71.96, p<0.001). In addition, ACE activity was significantly increased in CKD5D as compared to CKD3-5 (4454.48±87.10 versus 4181.65±58.37, p=0.001) (Figure 3A). In concordance, when paired case-control studies were performed, same results were observed (Figure 3B).

Circulating ACE activity was increased in females as compared to males in CONT and CKD3-5 (p<0.001). However, no differences were observed in CKD5D (p=0.057)(Table 3).

CONT with presence of plaques showed decreased levels of circulating ACE activity as compared to those without plaques (p=0.011). However, no differences were observed in CKD3-5 and CKD5D. ACE activity was decreased in CKD3-5 with hypertension or dyslipidemia as compared to non-hypertensive (p=0.001) or without dyslipidemia (p=0.004) (Table 3). We found a significant indirect correlation between circulating ACE activity, age (p=0.033) and glycosylated hemoglobin (p=0.019) in CONT.

ACE activity was increased in CONT and CKD3-5 in ARBs therapy as compared to non-treated patients. As expected, subjects treated with ACEi had lower levels of ACE activity as compared to non-treated subjects in all groups. ACE activity was decreased in CKD3-5 and CKD5D treated with cholecalciferol as compared to non-treated (Table 3).

By multiple regression analysis (Table 4), female gender and younger age were identified as independent predictors of circulating ACE activity in CONT and CKD3-5. Diabetes was also identified as an independent predictor of circulating ACE activity in CKD3-5. As well as in the bivariate analysis, ACEi therapy was inversely associated with ACE activity in all studied groups. Furthermore, cholecalciferol treatment was found as an independent predictor of ACE activity in CKD3-5 and CKD5D. ARBs therapy was identified as an independent predictor of circulating ACE activity in CONT.

Discussion

The present study demonstrates that circulating ACE2 and ACE activities may be measured in human EDTA plasma. This is the first study showing that circulating ACE2 activity is decreased

in CDK3-5 and dialysis patients without previous history of CV disease. In addition, we also showed that ACE2 correlates with the classical CV risk factors namely male gender, advanced age and diabetes in CKD3-5, and male gender and advanced age in CKD5D.

ACE2 enzymatic activity has been widely studied in renal, heart and other tissues under physiological and pathological conditions [21, 22]. However, few studies have assessed human circulating ACE2 activity, and the majority of them measured ACE2 activity in serum or heparin blood samples [12, 13]. We previously measured circulating ACE2 activity in serum from KT and acute myocardial infarction patients [14, 17]. Initially, we were not able to measure ACE2 activity in EDTA plasma samples. The chelating agent EDTA completely inhibits tissue ACE2 and soluble secreted ACE2 from CHO cell media activity, by chelating the zinc ion required for the metalloprotease activity [22, 25]. Hence, we made an effort to measure ACE2 activity in EDTA plasma samples. We demonstrated by adding zinc chloride, and subsequently avoiding the EDTA chelating effect that ACE2 and ACE activities could be measured.

Roberts et al. [18] demonstrated that plasma ACE2 activity was decreased in dialysis patients. In those patients, male gender and diabetes were associated with increased ACE2, while RAS blockade did not affect circulating ACE2. However, in their study the sample size was small and healthy subjects used for comparison were not contemporaneous with CKD patients. Here, we present a study with larger sample size (n=2572) and contemporaneous studied groups. Of note that in our study patients had no history of CV disease. In agreement with Roberts et al., initially we found circulating ACE2 activity decreased in dialysis patients. As expected, measurement of circulating ACE2 pre and post-dialysis showed no differences (data not shown), demonstrating that the enzyme is not removed by dialysis. One surmises that the hemodialysis itself could not alter the levels of the ACE2 activity in plasma owing to its large molecular size [26]. In our study, dialysis patients were younger than CKD3-5 and control groups. When paired case-control studies were performed, the differences among the CKD groups were not observed, suggesting that the decrease in ACE2 within the dialysis patients may be ascribed to age. Within CKD5D, CKD3-5 and CONT, a significant difference in the level of circulating ACE2 activity was demonstrated between males and females. Our results confirm the work of others, who showed that circulating ACE2 is sex-dependent, with higher levels in males [16-18]. Data from animal models suggest that soluble ACE2 shedding is stimulated by the tumor necrosis factor-α

convertase ADAM17 [27-29]. It is possible that the increase of ACE2 in males may be related to the increase of ADAM17 shedding. Further studies focused in the ADAM17/ACE2 axis and gender differences are needed to confirm this premise.

Many studies have associated circulating ACE2 activity with higher risk of CV disease. Epelman et al. [15] have previously demonstrated that circulating ACE2 activity is elevated in patients with diagnosis of heart failure. In recent studies from our group we showed that ACE2 activity is up-regulated in the acute phase of ST-elevation myocardial infarction and correlates with the infarct size [14]. Furthermore, KT patients with a previous history of ischemic heart disease presented increased ACE2 activity [17]. Soro-Paavonen et al. demonstrated that circulating ACE2 is increased in patients with diabetes and decreased eGFR or other vascular complications such as CV disease [16]. In concordance, in our study we demonstrated that ACE2 activity is also increased in diabetic CKD patients and it correlates with glycosylated hemoglobin. In mice with experimental diabetes, ACE2 activity is increased in the renal cortex and in the circulation, suggesting a potential mechanism to adapt to diabetes-associated AngII overactivity [21, 22]. As circulating ACE2 activity is increased starting at an early stage of diabetes and correlates with GFR, the measurement of ACE2 activity may become a new biomarker of CV disease in CKD.

Circulating ACE activity is increased in CKD3-5 and CKD5D without previous history of CV disease. We have demonstrated that ACE activity correlates with the classical CV risk factors such as male, advanced age and diabetes in CKD3-5. As expected, ACEi therapy was inversely associated with ACE activity in all groups. In concordance with our results, some studies have found lower levels of circulating ACE in subjects with a history of hypertension [12] and higher levels in diabetic patients with renal complications [30]. However, other studies have not found relationship between circulating ACE and the classical CV risk factors [17, 31, 32]. We surmise that the incongruences observed between studies and populations may be ascribed to the effect of RAS blockade on circulating ACE. For ethical reasons, RAS blockade agents were not stopped for the study.

In conclusion, this study shows that circulating ACE2 and ACE activities can be recovered and detected in human EDTA-plasma samples by adding zinc chloride. In addition, ACE2 activity

directly correlated with the classical CV risk factors such as male gender, diabetes and older age. These findings may have therapeutic implications for CV disease and help to delay the progression of CKD. Prospective studies with a short and long-term follow-up will help us to elucidate the ACE2 role as a biomarker in CKD.

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Tables

Table 1. Clinical characteristics of study cohorts.

					P-values		
	Total population	CONT	CKD3-5	CKD5D	CONT vs CKD3-5	CONT vs CKD5D	CKD3-5 vs CKD5D
Age (years)	56.8±12.65	54.3±11.49	59.5±11.86	52.86±13.77	p<0.001	p=0.094	p<0.001
Male/Female	1555/1017	347/272	1093/662	409/279	p=0.020	p=0.632	p=0.593
Diabetes	584 (22.7%)	76 (12.3%)	506 (28.8%)	124 (18.0%)	p<0.001	p=0.038	p<0.001
Hypertension	2006 (78%)	248 (40.1%)	1588 (90.5%)	590 (85.8%)	p<0.001	p<0.001	p=0.009
Dyslipidemia	1491 (58%)	231 (37.3%)	1215 (69.2%)	363 (52.8%)	p<0.001	p<0.001	p<0.001
Smoking	512 (19.9%)	128 (20.7%)	337 (19.2%)	138 (20.1%)	p=0.149	p=0.091	p=1
Body weight (kg)	76.4±15.23	77.2±15.03	78.0±14.85	72.40±15.70	p=0.642	p<0.001	p<0.001
Glycosylated hemoglobin (%)	5.9±1.19	5.7±1.04	6.3±1.30	5.51±1.07	p<0.001	p=0.218	p<0.001
Glomerular Filtration Rate (ml/min/1.73m2)	48.3±29.81	89.5±17.66	32.6±13.86	-	p<0.001	-	-
ACEi treatment	680 (26.4%)	64 (10.3%)	601 (34.2%)	130 (18.9%)	p<0.001	p=0.001	p<0.001
ARB treatment	1116 (43.4%)	145 (23.4%)	994 (56.6%)	219 (31.8%)	p<0.001	p=0.004	p<0.001
Insulin treatment	316 (12.3%)	11 (1.8%)	281 (16.0%)	219 (31.8%)	p<0.001	p<0.001	p=0.400

Continuous variables are expressed as means ± SD and categorical variables are represented by the number and the percentage of patients. CONT, control patients; CKD3-5, non-dialysis patients with chronic kidney disease stage 3-5; CKD5D, dialysis patients; ACEi, inhibitors of angiotensin converting enzyme; ARB, angiotensin II receptor blockers.

Table 2. Intraclass Correlation Coefficients and percentage of recovery for ACE2 and ACE activity between different sample conditions.

	Intraclass Correlation Coefficient (95% Confidence Interval)	
	ACE2 Activity	**ACE Activity**
EDTA-plasma ZnCl2 sample vs Serum sample	**0.95** (0.91 - 0.97)	**0.90** (0.90 - 0.95)
Serum sample vs Plasma-Heparin sample	**0.95** (0.91 - 0.98)	**0.93** (0.86 - 0.97)
EDTA-plasma ZnCl2 sample vs Plasma-Heparin sample	**0.97** (0.94 - 0.98)	**0.84** (0.68 - 0.92)
	% Recovery	
EDTA-plasma ZnCl2 sample vs Serum sample	107%	89%
EDTA-plasma ZnCl2 sample vs Plasma-Heparin sample	91%	83%

Table 3. Influence of different variables and treatments on circulating ACE2 and ACE activity in each studied group.

		Circulating ACE2 activity (RFU/µl/h)±SEM			Circulating ACE activity (RFU/µl)±SEM		
		CLINICAL VARIABLES					
		CONT	CKD3-5	CKD5D	CONT	CKD3-5	CKD5D
Gender	Male	61.4±2.26	50.6±1.53	45.6±2.45	3541.2±94.8	4032.8±71.8	4574.9±109.9
	Female	42.5±1.61*	36.7±1.47*	27.7±1.37*	4144.1±106.9*	4426.9±98.6*	4270.0±141.8
Diabetes	No	51.8±1.59	43.4±1.35	37.1±1.43	3766.5±75.4	4103.5±66.7	4459.7±97.2
	Yes	62.0±4.30*	50.3±1.97*	45.0±6.06	4127.7±230.3	4378.0±117.7	4431.1±196.3
Hypertension	No	47.2±1.30	43.2±2.87	36.7±4.05	3825.9±89.6	4669.2±189.6	14666.3±188.5
	Yes	62.0±3.18*	45.6±1.20	38.8±1.76	3782.5±120.5	4130.8±61.2*	4418.1±96.8
Dyslipidemia	No	49.5±1.61	43.6±1.55	33.9±1.30	3806.3±90.0	4404.8±105.8	4369.9±118.1
	Yes	59.0±2.97*	46.2±1.47	42.7±2.83*	3814.1±120.1	4080.2±69.8*	4531.4±127.0
Smoking	No	51.7±1.49	44.5±1.19	38.0±1.74	3817.8±80.7	4229.4±65.2	4414.2±94.4
	Yes	57.8±4.40	49.4±2.96*	40.5±4.02	3775.8±159.7	3976.7±130.5	4599.4±212.3
Plaques	Absence	48.9±2.29	39.7±1.89	31.2±1.70	3995.0±106.0	4159.6±99.9	4310.2±157.8
	Presence	56.7±1.94*	48.0±1.38*	41.8±2.19*	3638.5±97.0*	4191.7±71.8	4518.1±104.4
		TREATMENTS					
		CONT	CKD3-5	CKD5D	CONT	CKD3-5	CKD5D
ACEi	No	52.5±1.59	46.2±1.48	38.9±1.92	3908.4±75.5	4993.8±70.3	4945.9±87.5
	Yes	57.4±4.33	44.0±1.65	37.2±2.38	2889.8±203.0*	2694.7±64.6*	2410.6±145.4*
ARB	No	49.7±1.27	43.7±1.49	37.7±2.17	3719.6±80.3	3729.7±85.2	4367.3±102.4
	Yes	64.2±4.89*	46.7±1.62	40.3±1.99*	4116.3±158.3*	4535.1±77.8*	4647.1±163.8
Oral antidiabetic drugs	No	51.9±1.51	44.2±1.19	38.6±1.64	3795.0±75.2	4165.4±61.9	4464.7±88.1
	Yes	63.1±4.89*	54.6±3.31*	35.1±5.86	3937.7±245.2	4307.7±175.2	3954.3±582.9
Insulin	No	52.9±1.5	44.7±1.26	37.3±1.49	3805.3±72.1	4112.8±61.6	4470.8±94.4
	Yes	59.6±11.85	48.9±2.17*	45.5±6.84	4087.1±848.3	4549.9±168.3	4356.9±227.5
Cholecalciferol	No	53.0±1.50	45.6±1.15	39.0±1.66	3805.0±72.0	4214.4±59.7	4492.7±88.3
	Yes	56.9±0.00	37.9±4.51	25.0±3.94*	6215.3±0.00	3211.4±229.2*	3250.6±438.7*

CONT, control patients; CKD3-5, non-dialysis patients with chronic kidney disease stage 3-5; CKD5D, dialysis patients; ACEi, inhibitors of angiotensin converting enzyme; ARB, angiotensin II receptor blockers.
*p<0.05 (No vs Yes, Male vs Female and Absence vs Presence)

Table 4. Multiple linear regression analysis of potential predictors of circulating ACE2 and ACE activities.

PREDICTORS OF CIRCULATING ACE2 ACTIVITY			
		Standardized coefficient (β)	**p-value**
a) CONT			
	Male	0.243	<0.001
	Advanced age	0.148	<0.001
b) CKD3-5			
	Male	0.224	<0.001
	Advanced age	0.060	0.020
	Diabetes	0.074	0.004
c) CKD5D			
	Male	0.318	<0.001
	Advanced age	0.119	0.003
	ARB treatment	0.095	0.020
	Cholecalciferol treatment	-0.095	0.018
PREDICTORS OF CIRCULATING ACE ACTIVITY			
		Standardized coefficient (β)	**p-value**
a) CONT			
	Male	-0.182	<0.001
	Advanced age	-0.087	0.035
	ACEi treatment	-0.152	<0.001
	ARB treatment	0.124	0.003
b) CKD3-5			
	Male	-0.062	0.004
	Advanced age	-0.069	0.001
	Diabetes	0.071	0.001
	ACEi treatment	-0.562	<0.001
	Cholecalciferol treatment	-0.074	0.001
c) CKD5D			
	ACEi treatment	-0.580	<0.001
	Cholecalciferol treatment	-0.087	0.012

Data are expressed as regression coefficients and p values. Dependent variables: circulating ACE2 activity, expressed in LnACE2 and circulating ACE activity, expressed in LnACE.

CONT, control patients; CKD3-5, non-dialysis patients with chronic kidney disease stage 3-5; CKD5D, dialysis patients; ACEi, inhibitors of angiotensin converting enzyme; ARB, angiotensin II receptor blockers.

Legends to figures

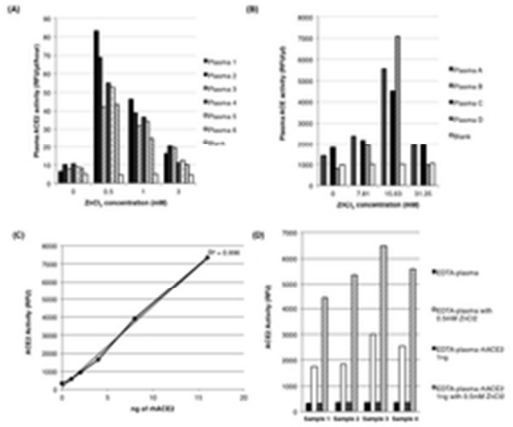
Figure 1. **Circulating ACE2 (A) and ACE (B) enzymatic activity at increased concentrations of Zinc Chloride ($ZnCl_2$) (C) Standard curve of recombinant human ACE2 (rhACE2). (D) ACE2 activity in EDTA-plasma samples with the rhACE2 and $ZnCl_2$**. (A) ACE2 activity was assessed in 6 different plasma samples (1-6) using the following concentrations of $ZnCl_2$: 0, 0.5, 1 and 3 mM. (B)ACE activity was assessed in 4 different plasma samples (A-D) using the following concentrations of $ZnCl_2$: 0, 7.81, 15.63 and 31.25 mM. (C) ACE2 activity was linearly increased with increasing the rhACE2 concentrations. (D) ACE2 activity was assessed in 4 EDTA-plasma samples with the following conditions: EDTA-plasma alone; EDTA-plasma adding $ZnCl_2$ at a concentration of 0.5 mM; EDTA-plasma adding 1 ng of rhACE2; and EDTA-plasma adding both $ZnCl_2$ at 0.5 mM and 1 ng of rhACE2.

Figure 2. **Circulating ACE2 activity between studied groups**. (A) ACE2 activity was significantly decreased in CKD3-5 (grey bars) and CKD5D (white bars) patients as compared to CONT (black bars) (*p<0.001). CKD3-5 showed an increase in plasma ACE2 activity as compared to CKD5D ($^{\$}$p<0.001). When matching samples with an equal distribution of gender, diabetes, hypertension, dyslipidemia, smoking habits, weight and age (B), ACE2 activity was significantly decreased in CKD3-5 and CKD5D as compared to CONT but no differences were found between CKD3-5 and CKD5D (p=0.27).

CONT, control patients; CKD3-5, non-dialysis patients with chronic kidney disease stage 3-5; CKD5D, dialysis

Figure 3. Circulating ACE activity between studied groups. (A) ACE activity was increased in CKD3-5 (grey bars) (*p=0.035) and in CKD5D (white bars) (*<0.001) as compared to CONT (black bars). CKD5D showed an increase in plasma ACE activity as compared to CKD3-5 ($^{\$}$p=0.001). When assessing a paired case-control study (B), same results were obtained.

CONT, control patients; CKD3-5, non-dialysis patients with chronic kidney disease stage 3-5; CKD5D, dialysis



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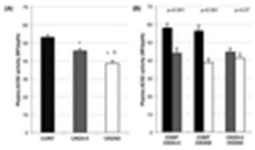

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CONT, control patients; CKD3-5, non-dialysis patients with chronic kidney disease stage 3-5; CKD5D, dialysis

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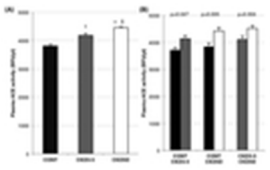

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CONT, control patients; CKD3-5, non-dialysis patients with chronic kidney disease stage 3-5; CKD5D, dialysis

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