Title: **Circulating Angiotensin Converting Enzyme 2 activity as a biomarker of silent atherosclerosis in patients with chronic kidney disease.**

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Abstract (249 words)

Background. Circulating Angiotensin Converting Enzyme 2 (ACE2) activity in chronic kidney disease (CKD) patients without previous history of cardiovascular disease (CVD) has been associated with classical risk factors (advanced age, diabetes and male gender). Furthermore, silent atherosclerosis has been described as a pathological link between CKD and CVD. We analyzed baseline ACE2 activity in non-dialysis CKD stages 3-5 (CKD3-5) patients as a biomarker of renal progression, silent atherosclerosis and cardiovascular events after 2 years of follow-up.

Methods. Prospective study of 1458 CKD3-5 subjects without any previous cardiovascular event included in the Spanish multicenter NEFRONA study. Association between baseline circulating ACE2 activity and renal parameters, carotid/femoral echography, atheromatous disease, ankle brachial index, intima-media thickness, need of renal replacement therapy, cardiovascular events and mortality at 24 months of follow-up were analyzed.

Results. Patients with an increase in the number of territories with plaques at 24 months showed significantly higher levels of baseline ACE2 activity as compared to stable patients (46.90±1.63 vs 38.73±1.59, $p<0.001$). Multivariate linear regression analysis showed that pathological ankle-brachial index and progressive silent atherosclerosis defined as an increased number of territories with plaques at 24 months were associated with increased baseline ACE2 activity. Male gender, older age, diabetes and increased baseline circulating ACE2 activity were independent predictors of atherosclerosis at 24 months of follow-up.

Conclusions. In CKD3-5 patients, higher circulating ACE2 activity at baseline is associated with higher risk for silent atherosclerosis, suggesting that ACE2 activity may serve as a biomarker to predict cardiovascular risk before CVD is established.

Introduction

Cardiovascular disease (CVD) is the leading cause of morbidity and mortality in patients with chronic kidney disease (CKD) [1-3]. The risk of death from any cause increases as the estimated GFR declines [2] and patients with end-stage renal disease (ESRD) have a rate of mortality that exceeds the need of renal replacement therapy [4]. Silent atherosclerosis has been described as a pathological link between CKD and CVD and could be a useful marker for predicting adverse cardiovascular outcomes in CKD patients[5, 6]. In CKD patients, carotid intima media thickness (IMT) has been demonstrated to be increased as compared to healthy patients [7, 8] and to be an independent predictor of all-cause and cardiovascular mortality [9]. In addition, carotid plaque largely increases the risk of CV events [10]. Recent results from the multicenter prospective observational NEFRONA study [11], that includes CKD patients and controls without previous cardiovascular events, have shown that the prevalence of atheromatous plaques is higher among CKD patients, correlating with the severity of CKD. Interestingly, the highest prevalence is observed among dialysis patients [12, 13].

The renin-angiotensin system (RAS) plays also a major role in the pathophysiology of CVD and renal injury[14, 15]. Within the RAS, angiotensin-converting enzyme 2 (ACE2) [16] acts as a monocarboxypeptidase that cleaves the C-terminal aminoacid of angiotensin II (AngII) to generate the peptide Ang1-7 which counteracts the adverse effects of AngII [17]. ACE2 is an integral cell membrane protein that can undergo cleavage or shedding to release the catalytically active ectodomain into the circulation [18]. Circulating ACE2 levels were at first only detected in subjects with advanced age and with higher prevalence of CVD, diabetes and hypertension [19]. Subsequent studies have confirmed that circulating ACE2 activity can be detected in healthy subjects [20] and that it is increased in heart failure patients, acute myocardial infarction [21, 22], diabetes [23], kidney transplant patients [24], and in pre-dialysis CKD patients [25]. We have recently shown that in CKD patients without previous history of CVD circulating ACE2 activity directly correlates with the classical cardiovascular risk factors such as male gender, older age and diabetes [26]. It has also been demonstrated a protective role of ACE2 against atherosclerosis in experimental studies. ACE2 overexpression has been shown to inhibit the development of early atherosclerotic lesions [27, 28] and to enhance the stability of atherosclerotic

plaques [29] partly due to counter regulation of AngII signaling and inhibition of inflammatory response. In concordance, ACE2 deletion increases the development of atherosclerosis in fat-fed *Ldlr*$^{-/-}$ mice [30] and plaque accumulation in atherosclerosis-prone apolipoprotein E knockout (*ApoE* KO) mice [31].

We have previously shown that circulating ACE2 activity in CKD patients without previous history of CVD from the NEFRONA study directly correlates with classical CV risk factors namely older age, diabetes and male gender[26]. We now hypothesized that baseline ACE2 activity in CKD stages 3-5 (CKD3-5) patients serves as a biomarker of renal progression, atherosclerosis and CV events after 24 months of follow-up.

1. Subjects and Methods

1.1. Patients and Variables

Study population included 1458 non-dialysis CKD3-5 subjects from the observational and multicenter study (NEFRONA project), recruited from October 2009 to June 2011 [11, 26] and with 24 months of follow-up. This prospective study included male and female patients without history of CVD (angina pectoris, acute myocardial infarction, ischemic stroke, hemorrhagic stroke, abdominal aortic aneurysm and atherosclerosis), and ages ranged between 18 and 74 years old. Exclusion criteria were pregnancy, VIH infection, any type of organ transplantation, previous history of carotid artery disease, active infections, any hospitalization in the last month, and intercurrent illness that presumes absence of follow-up or survival expectation less than 1 year.

Baseline clinical variables, treatment profile, analytical variables, presence of plaques and ACE2 enzymatic activities have been previously reported [26, 32]. For the prospective study at 24 months, renal parameters (serum creatinine and glomerular filtration rate), carotid/femoral echography, atheromatous disease (AD), ankle brachial index (ABI), intima-media thickness (IMT), need of renal replacement therapy (kidney transplantation or dialysis), cardiovascular events and mortality were assessed (Figure 1).

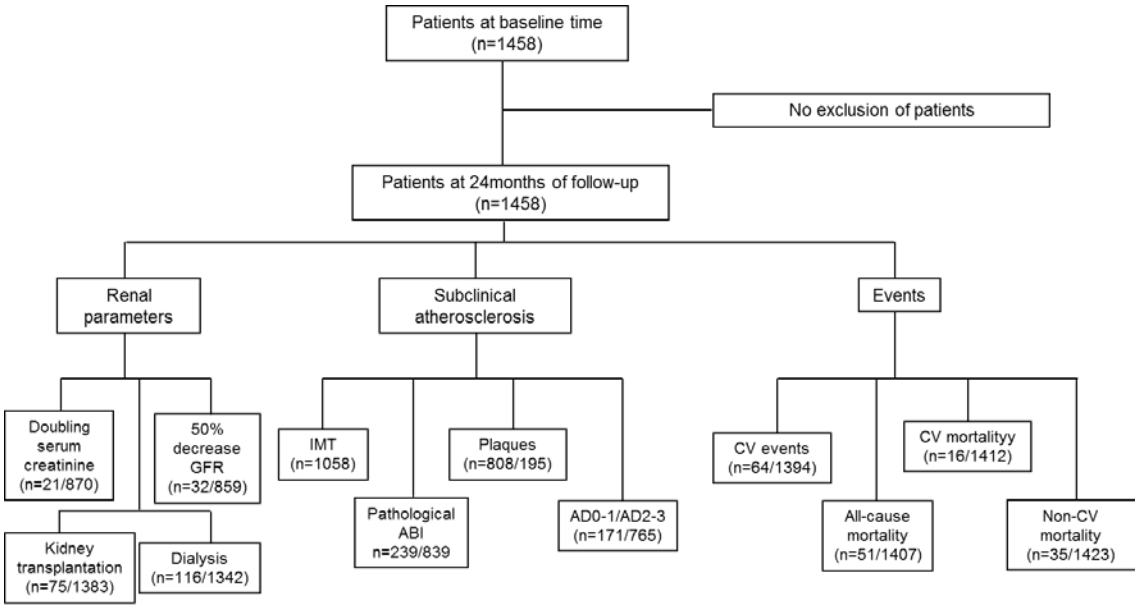

Figure 1. Schematic representation of patients analyzed in the study*. For the analysis of renal parameters and subclinical atherosclerosis some patients were lost. Exclusion criteria during the 24 months of follow-up were patients in need of renal replacement therapy (dialysis or kidney transplant), all-cause of mortality, cardiovascular events, having an atheromatous disease at stage 3 (AD 3) or transfer to another hospital.*

1.2. *Renal parameters*

Progression of renal disease was evaluated according to doubling of serum creatinine or 50% decrease in estimated glomerular filtration rate (eGFR) calculated with the modification of diet in renal disease (MDRD)-4 formula at 24 months. Renal replacement therapy was defined as kidney transplantation or dialysis during the 24 months of follow-up. For the assessment of doubling of serum creatinine and the decrease in eGFR, patients that started dialysis or had a kidney transplant during the 24 months of follow-up were excluded.

1.3. *Silent Atherosclerosis*

Subclinical atherosclerosis was evaluated as described previously [11]. Participants underwent a carotid and femoral ultrasound to measure IMT, using the Vivid BT09 apparatus (General Electric instrument) equipped with a 6–13 MHz broadband linear array probe. The analysis of

the presence of atheromatous plaques was performed by a unique reader in a blinded fashion, using the semi-automatic software EchoPAC Dimension (General Electric Healthcare). To assess the quality of the reading and the intraobserver reliability, a sample of 20 individuals was measured 3–5 times on different days. A kappa coefficient of 1 was obtained, indicating excellent intraobserver reliability.

Plaque presence was evaluated in a total of 10 territories: right common carotid arteries, right carotid bulb, right internal carotid arteries, left common carotid arteries, left carotid bulb, left internal carotid arteries, right common femoral arteries, right superficial femoral arteries, left common femoral arteries, and left superficial femoral arteries.To assess the evolution of plaques from baseline time to 24 months, patients were classified in three groups: no plaque (patients without plaque both at baseline and 24 months); *de novo* plaque (appearance of plaque at 24 months); and baseline plaque (plaques both at baseline and 24 months). Furthermore, a classification according the location of plaques was performed: any plaque (presence of plaque in any territorie); carotid plaque (presence of plaque in carotid territories exclusively????); and femoral plaque (presence of plaque in femoral territories exclusively????);. Patients were classified in three groups according to the number of territories with plaques: 0; 1 to 4; and ≥5. The increase in number of territories with plaques from baseline to 24 months was also determined.

Vascular Doppler MD2 Hungleigth was used with an 8 MHz transducer and a sleeve for making manual blood pressure. The determination of blood pressure was performed in the brachial artery in both arms and in both feet. To calculate the ABI, the higher brachial blood pressure was used or the closest in time to the malleolar measure. A pathological ABI was defined as a value ≤0.9, diagnostic of a limb ischemia, or ≥1.4, diagnostic of arterial incompressibility and stiffness, usually ascribed to vascular wall calcification.

AD was scored into 2 groups according to the ultrasonography findings and the ABI measurements: Stage 0-1 (AD 0-1), that included subjects with ABI ≥0.7 and/or carotid IMT ≥90% according to reference range (RR), and Stage 2-3 (AD 2-3), that included patients with ABI <0.7 and/or carotid plaque without or with stenosis >50%.

1.4. *Events*

Events were classified as cardiovascular events, cardiovascular mortality, non- cardiovascular mortality, and all-cause mortality (both cardiovascular and non- cardiovascular). Cardiovascular events included angina pectoris, acute myocardial infarction, ischemic stroke, cerebral infarction, subarachnoid hemorrhage, intracerebral hemorrhage, cardiac insufficiency, atherosclerosis of extremities with intermittent claudication and abdominal aortic aneurysm. Causes of cardiovascular mortality were defined as: myocardial ischemia and infarction, hyperkalemia or arrhythmia, cerebrovascular accident (ischemic or hemorrhagic), hemorrhage due to aneurysm rupture, mesenteric infarct and sudden death. Non- cardiovascular mortality causes included infection, neoplasia, accident, renal, uremic, non-determined or unknown [33].

1.5. *Statistical Analysis*

Normality of the continuous variables was assessed by normal probability plots. Variables were expressed as mean±SE. Continuous variables were evaluated by the ANOVA or the non-parametric Mann-Whitney test. Bivariate correlations were calculated by the Spearman's correlation coefficient. Multiple linear regression analyses, using the natural logarithmic transformation of baseline circulating ACE2 activity or the number of territories with plaques at 24 months as dependent variables, were performed to identify independent predictors of these variables. These analyses were adjusted by age, gender and diabetes, which have been previously described as independent predictors of increased circulating ACE2 activity in CKD3-5 patients [26]. *SPSS* version 18.0 for Windows was used for statistical calculations. $P<0.05$ was considered statistically significant.

2. Results

2.1. *ACE2 activity and renal function*

Baseline circulating ACE2 activity was slightly increased in patients that doubled serum creatinine as compared with stable patients (55.66±9.58 RFU/μL/h versus 43.04±1.27, p=0.154). There were no differences between decrease in 50% of eGFR and baseline circulating ACE2 (52.17±6.86 versus 43.01±1.28, p=0.132). There were no differences in baseline circulating ACE2 between patients that needed renal replacement therapy (kidney transplant or dialysis) and patients that maintained kidney function (41.11±3.22 versus 45.55±1.17, p=0.792 and 52.03±5.20 versus 44.75±1.13, p=0.446; respectively).

2.2. ACE2 activity and atherosclerosis

The relationship between baseline circulating ACE2 levels and subclinical atherosclerosis was studied. The association of baseline circulating ACE2 activity and evolution of plaques followed the same pattern in the any plaque (Figure 2A) and femoral plaque (Figure 2B) groups: patients with *de novo* plaque (any plaque: 41.97±2.44; femoral plaque: 46.62±3.19) and baseline plaque (any plaque: 48.14±1.71; femoral plaque: 48.85±1.90) showed higher levels of baseline circulating ACE2 activity as compared to patients with no plaque (any plaque: 34.04±1.55, p=0.007; femoral plaque: 35.89±1.50, p=0.002). In addition, levels of baseline ACE2 activity were significantly higher in patients with baseline plaque as compared to *de novo* plaque (p<0.001 for both any plaque and femoral plaque groups). For carotid plaque only a significant difference between no plaque and baseline plaque was found (38.37±1.32 versus 48.90±2.10, p<0.001) (Figure 2C). We also determined the levels of baseline circulating ACE2 activity according to the number of territories with plaques (maximum of 10 territories) at 24 months (Figure 2D). Patients with higher number of territories with plaques (1-4 and ≥5) had higher levels of baseline ACE2 activity (43.02±1.48 and 52.07±2.96, respectively) as compared to patients without plaque at 24 months (34.50±1.80, p<0.001 and p<0.001). Baseline ACE2 activity in patients with ≥5 territories was significantly higher as compared to patients with 1-4 territories (p=0.003). Furthermore, patients with an increase in the number of territories with

plaques at 24 months as compared to stable patients, showed significantly higher levels of baseline ACE2 activity (46.90±1.63 vs 38.73±1.59, p<0.001) (Figure 2E).

We found a significant but weak correlation between baseline ACE2 activity and IMT at 24 months (p=0.023). Furthermore, baseline circulating ACE2 activity was higher in patients with pathological ABI (defined as a value ≤0.9 or ≥1.4) as compared to patients with normal ABI (51.53±3.49 vs 42.13±1.20, p=0.002) (Figure 2F). Patients with severe AD (AD 2-3) showed increased levels of baseline circulating ACE2 activity as compared to those with incipient AD group (AD 0-1) (47.47±1.58 vs 35.74±1.83, p<0.001) (Figure 2G).

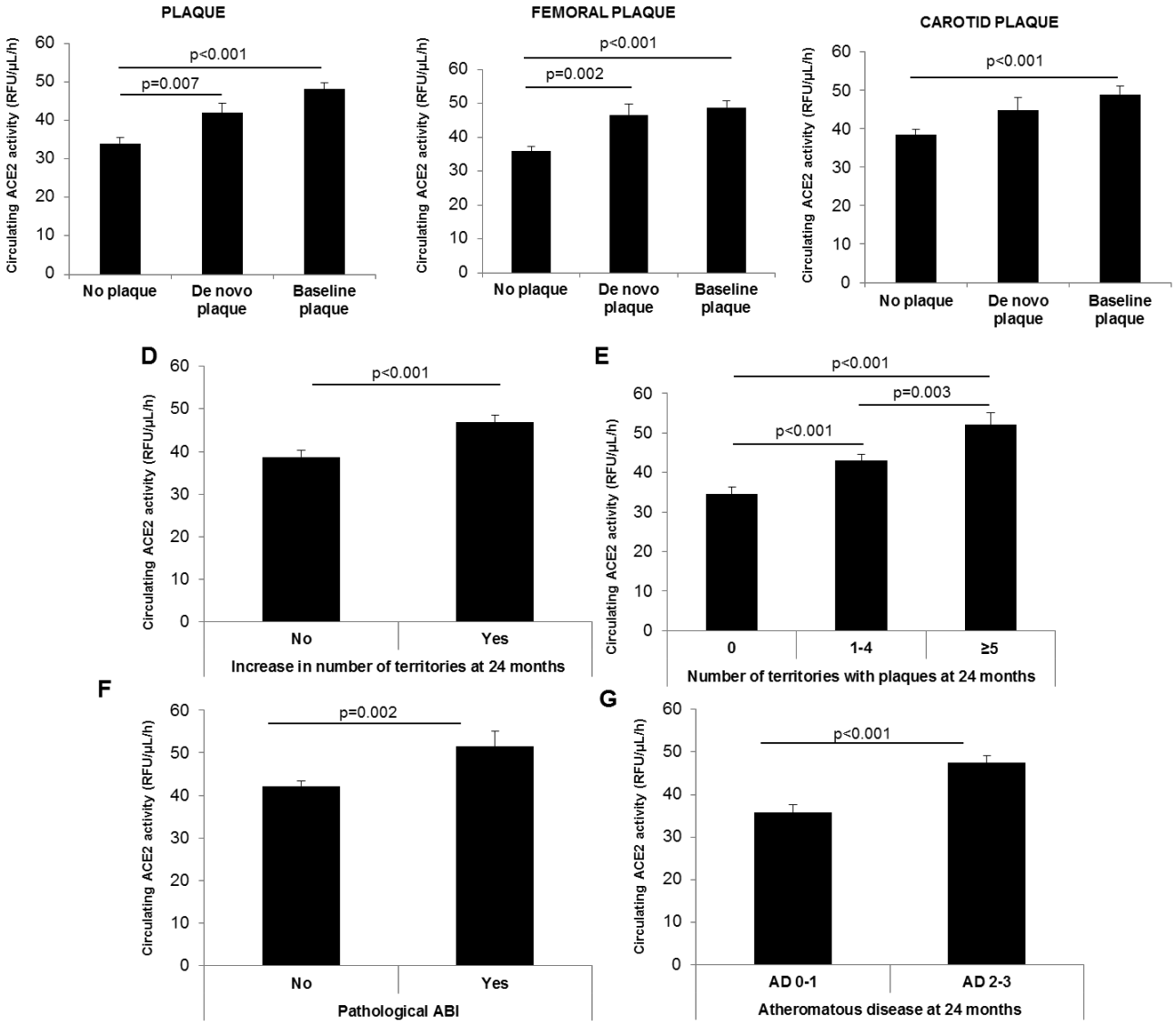

Figure 2. Association of plaques with baseline circulating ACE2 activity. A) Any Plaque*. Baseline circulating ACE2 activity was significantly increased in patients with de novo plaque (p=0.009) and baseline plaque (p<0.001) as compared to those patients with no plaque.* ***B) Femoral plaque****. Patients with femoral de novo plaque (p=0.002) and baseline plaque (p<0.001) had elevated levels of baseline ACE2 activity as compared to those patients with no plaque.* ***C) Carotid plaque****. Baseline circulating ACE2 activity was significantly increased in patients with baseline plaque as compared to those patients without plaque (p<0.001).* ***D) Number of territories with plaques at 24 months****. Baseline circulating ACE2 activity was significantly increased in Patients with higher number of territories with plaques at 24 months (1-4 and ≥5) (p=0.001 and p<0.001). Furthermore, patients with ≥5 territories had increased levels of baseline ACE2 activity as compared to patients with 1-4 territories with plaques (p=0.007).* ***E) Increase in number of territories with plaques at 24 months****. Patients were classified in two groups depending on whether they had increased the number of territories with plaques from baseline to 24 months or not. Baseline ACE2 activity was significantly elevated in patients that have increased the number of territories with plaques (p<0.001).* ***F) Association of subclinical atherosclerosis with baseline circulating ACE2 activity: Pathological ABI at 24 months****. Baseline circulating ACE2 activity was increased in patients with a pathological ABI at 24 months of follow-up.* ***G) Association of subclinical atherosclerosis with baseline circulating ACE2 activity: Atheromatous disease at 24 months****. Baseline circulating ACE2 activity was increased in patients with severe AD (AD 2-3) at 24 months as compared to patients in incipient stages of AD (AD 0-1) (p<0.001). ABI: ankle-brachial index; AD: atheromatous disease.*

Clinical data according to the number of territories with plaques at 24 months or the increase in number of territories are shown in Table 1. Male gender, diabetes, hypertension, dyslipidemia, older age and smoking habits were significantly increased in patients with territories with plaques as compared to patients without plaques. In addition, circulating ACE2 activity was higher as the number of territories increased. The percentage of patients with family history of CVD was also increased in those patients with territories, but it did not reach statistical significance. There was no difference between renal parameters and the number of territories

with plaque (Table 1). Clinical data regarding the increase in number of territories during the 24 months of follow-up showed the same pattern as mentioned above (Table 1).

Table 1. Clinical parameters according to the number of territories with plaques or the increase in the number of territories with plaques.

	Number of territories with plaques at 24 months			
	0	**1-4**	**≥5**	**p-value**
Gender (% male)	44.6	60.0	77.7	<0.001
Age (years)	47.1±13.7	60.5±9.5	65.6±7.5	<0.001
Diabetes (%)	14.9	23.1	38.7	<0.001
Hypertension (%)	86.2	93.2	96.8	<0.001
Dyslipidemia (%)	59.5	69.7	75.5	0.001
Family history of CVD (%)	9.2	10.6	7.7	0.388
Smoking habits (%)	40.0	53.8	72.3	<0.001
Doubling serum creatinine (%)	2.5	2.4	1.6	0.762
50% decrease in eGFR (%)	4.4	3.4	2.8	0.695
Baseline circulating ACE2 activity (RFU/µL/h)	34.5±25.1	43.0±33.1	52.1±52.1	<0.001
	Increase in number of territories with plaques			
	No	**Yes**		**p-value**
Gender (% male)	56.7	66.3		0.002
Age (years)	54.8±13.7	62.3±9.5		<0.001
Diabetes (%)	18.0	31.4		<0.001
Hypertension (%)	88.7	95.6		<0.001
Dyslipidemia (%)	64.2	72.5		0.006
Family history of CVD (%)	8.8	9.8		0.576

Smoking habits (%)	50.5	60.5	0.002
Doubling serum creatinine (%)	1.6	2.7	0.301
50% decrease in GFR (%)	3.5	3.5	0.975
Baseline circulating ACE2 activity (RFU/µL/h)	38.7±31.3	46.9±39.6	0.001

Values for categorical variables are given as percentage; values for continuous variable as mean ±SD. CVD: cardiovascular disease; eGFR: estimated glomerular filtration rate.

By multivariate linear regression analysis, with baseline circulating ACE2 activity as dependent variable and adjusting by age, gender and diabetes, pathological ABI and increased number of territories with plaques were independently associated with increased baseline circulating ACE2 activity (Table 2). To further confirm baseline circulating ACE2 as a potential biomarker of atherosclerosis at 24 months of follow-up, a multivariate linear regression analysis was performed with the number of territories with plaques at 24 months as dependent variable. The analysis was adjusted by age, gender and diabetes. Male gender, older age, diabetes and increased baseline circulating ACE2 activity were independent predictors of atherosclerosis at 24 months of follow-up (Table 2).

Table 2. Multivariate linear regression analysis

Model 1. Dependent variable: baseline circulating ACE2 activity (expressed in LnACE2)		
	Standardized coefficient (β)	**P-value**
Male	0.222	<0.001
Age	0.002	0.953
Diabetes	0.047	0.134

Pathological ABI at 24 months	0.066	0.038
Number of territories with plaques at 24 months	0.111	0.003

Model 2. Dependent variable: number of territories with plaques at 24 months of follow-up

	Standardized coefficient (β)	**P-value**
Male	0.193	<0.001
Age	0.434	<0.001
Diabetes	0.143	<0.001
Baseline circulating ACE2 activity	0.094	0.001

Model 1: multivariate linear regression analysis of potential predictors of baseline circulating ACE2 activity*. By multiple linear regression, and adjusting by gender, age and diabetes, pathological ABI and number of territories with plaques at 24 months were found as potential predictors of increased baseline ACE2 activity (R=0.301).* ***Model 2: multivariate linear regression analysis of potential predictors of territories with plaques at 24 months of follow-up****. When multiple linear regression was performed with the number of territories with plaques as the dependent variable, and also adjusting by gender, age and diabetes, we found that increased baseline ACE2 activity still remained as a potential predictor of increased number of territories with plaques (R=0.559).*

Data are expressed as regression coefficients and P-value. Dependent variable in model 1: baseline circulating ACE2 activity (expressed in LnACE2). Dependent variable in model 2: number of territories with plaques at 24 months. Adjusted by gender, age and diabetes.

ABI, ankle-brachial index.

2.3. *ACE2 activity and cardiovascular and non-cardiovascular events*

No differences were found regarding baseline circulating ACE2 activity, cardiovascular events and cardiovascular mortality during the follow-up. Baseline circulating ACE2 was significantly higher in non-CV and all-cause of mortality patients (Table 3).

Table 3. Association of events and mortality with baseline circulating ACE2 activity

		Baseline circulating ACE2 activity	p-value
Cardiovascular event	**No**	45.40±1.16	0.167
	Yes	43.63±3.33	
Cardiovascular mortality	**No**	45.40±1.13	0.705
	Yes	38.88±4.90	
Non-Cardiovascular mortality	**No**	44.80±1.12	0.013
	Yes	63.37±9.12	
All-cause mortality	**No**	44.96±1.16	0.023
	Yes	51.03±4.40	

Values are expressed as mean±SD

3. Discussion

In our study we analyze the association between baseline circulating ACE2 activity, silent atherosclerosis, renal function progression and cardiovascular events in a subpopulation of the NEFRONA study during 24 months of follow-up. We found that in non-dialysis CKD3-5 patients elevated baseline circulating ACE2 activity is associated with increased risk for silent atherosclerosis. In CKD3-5 patients, atheromatosis progression is associated with male gender, older age, diabetes and baseline circulating ACE2 activity. These results suggest that ACE2 activity may serve as a biomarker to predict cardiovascular risk before CVD (angina pectoris, ischemic stroke or infarction) is established.

Studies in the NEFRONA population have been focused on the prevalence of silent atherosclerosis in CKD patients as compared to healthy population. It has been demonstrated that the prevalence of atheromatous plaques is increased in CKD population and that the highest prevalence is observed among dialysis patients[12, 13, 34]. As expected, the prevalence rates of severe carotid AD were more than two fold higher in patients with diabetic nephropathy as compared to those with any other causes of kidney disease. Moreover, the prevalence of silent AD increases with the severity of CKD, confirming previous findings in the whole cohort [12, 35]. Thus, patients with diabetes-induced renal disease are more likely to have endothelial damage beyond the kidneys. Patients with diabetic nephropathy at any grade of CKD are at particularly high risk of subclinical severe AD compared to any other causes of CKD. The use of carotid ultrasound can help in the early detection of AD, particularly in CKD population, in whom the classical risk scoring has been proven inefficacious. In addition, Martin et al. have also shown that high serum phosphate levels are associated with higher risk of atherosclerosis, expressed as the presence of plaques detected by ultrasound [36].

Recently, atheromatosis progression has been assessed in the NEFRONA population during 24 months of follow-up. They found four main findings: (1) a high prevalence of CKD patients with atheromatous plaque in carotid and/or femoral territories; (2) the only variables with a homogeneous association with the progression of plaque across CKD stages were the presence of diabetes and two interactions of age with ferritin and the presence of plaque at baseline; (3) risk factors predicting atheromatosis progression are different depending on the CKD stage; (4) and the progression of atheromatosis is associated with the progression of CKD. Consequently, they support the utility of the determination of atheroma plaque presence by arterial ultrasound as a powerful tool to predict atheromatosis progression in patients with CKD. Our study suggests that baseline ACE2 activity may predict 24-month atheromatosis progression measured by the increased number of territories with plaques in patients with non-dialysis CKD.

The RAS is a major hormonal system involved in the pathophysiology of CVD. ACE2 counterbalances the vasoconstrictor adverse effects of AngII by converting it to Ang1-7 [17]. Thus, several studies have proposed ACE2 as an emergent biomarker for CVD and it may become a

therapeutic target for atherosclerotic lesions. Initial studies in a large cohort were able to detect circulating ACE2 activity only in patients with CVD[19]. Subsequently studies demonstrated that circulating ACE2 activity can be detected both in patients with CVD and in healthy subjects[20] and that it was increased in heart failure patients[21, 37]. In concordance, we have shown that ACE2 activity is up-regulated in the acute phase of ST-elevation myocardial infarction and that it correlates with the infarct size[22]. Furthermore, in kidney transplant patients with previous history of ischemic heart disease we have observed that circulating ACE2 activity was increased[24]. In experimental studies, circulating and renal cortex ACE2 is increased in diabetic mice, suggesting a potential mechanism to adapt to diabetes-associated Ang II overactivity[38]. In addition, a cumulative damage from Ang II, that caused an age-dependent cardiomyopathy, was observed in mice with deletion of ACE2[39]. Therefore, circulating ACE2 activity may serve as a biomarker for CV risk. In agreement with these previous findings our results now suggest that circulating ACE2 activity may be a surrogate marker of CV risk by predicting the silent atheromatosis disease.

Soro-Paavonen et al. determined that ACE2 activity was increased in patients with diabetes, vascular complications and decreased eGFR[23]. In dialysis patients, males had increased levels of circulating ACE2 activity as compared to females[25]. In agreement, we have also previously demonstrated that ACE2 activity is increased in CKD males as compared to females. In CKD3-5 patients baseline circulating ACE2 activity correlated with the classical cardiovascular risk factors, such as male gender, advanced age and diabetes, while in dialysis patients it correlated with male gender and advanced age[26]. In experimental studies circulating ACE2 has been shown to be increased in diabetic mice. This increase was blunted by the administration of insulin (and glucose levels normalization) and the administration of vitamin D analog (without changes in glucose levels)[32, 38]. Giving these results one may surmise that the increase in circulating ACE2 activity may be an indicator of increased risk of CVD. Certain therapies aimed to decrease cardiovascular risk appear to modify circulating ACE2 activity.

Zulli et al. described that ACE2 was present on the endothelial cell layer overlying neo-intima and atherosclerotic lesions from thoracic aorta in rabbits. Specifically, they identified that a high proportion of macrophages and smooth muscle actin-positive cells within atherosclerotic

plaques expressed ACE2[40]. In concordance with these findings, ACE2 is also expressed in human atherosclerotic plaques and located in different cell types present in the lesion, such as endothelial cells and macrophages[41]. Early atherosclerotic lesions have been described to involve endothelial cell dysfunction, vascular smooth muscle cells (VSMC) proliferation and migration and endothelial cell matrix deposition[27]. Several experimental studies, including animal models and endothelial and VSMC, have shown that ACE2 activation improves endothelial repair and regeneration and, as a consequence, reduces atherosclerosis[27, 42]. In addition, it has been described that inhibition of AngII-induced inflammation by ACE2 overexpression may decrease endothelial dysfunction and, therefore, inhibit early atherosclerotic lesions[28] and enhance stability of atherosclerotic plaques[29]. In our study, we demonstrated that an increase of the number of territories with plaques at 24 months of follow-up is independently associated with elevated baseline circulating ACE2 activity in CKD3-5 patients. Thus, increased circulating ACE2 may activate circulating mechanisms that will lead to plaque formation and progression.

We also studied if circulating ACE2 activity at baseline may serve as a potential predictor of CV events and mortality over 2-years of follow-up in CKD3-5 patients. Surprisingly, we did not find differences between baseline ACE2 activity and cardiovascular events or cardiovascular mortality. We ascribe the lack of differences to the short-term follow-up and to the low number of cardiovascular events observed during the 24 months of follow-up in patients without previous history of CVD. Therefore, we surmise that a longer term follow-up will help us to better elucidate the role of ACE2 as a biomarker of CVD in our study population. There are no previous studies that have explored the association between circulating ACE2 activity and renal function progression. We did not find any significant difference in patients that doubled serum creatinine or had a 50% decrease in eGFR at 24 months of follow-up versus stable patients. Again, the lacking of significant differences may be related to the low number of patients with renal endpoints (doubling serum creatinine and/or 50% decrease in eGFR) and to the short-term follow up.

In conclusion, our study shows that baseline ACE2 activity may serve as a potential predictor for the development of silent atheromatosis in CKD patients without previous history of CV

disease. The strength of this association and the potential of baseline circulating ACE2 for predicting future cardiovascular events and mortality need longer follow-up studies.

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