Background. Patients with cardiovascular (CV) disease have an increased circulating angiotensin-converting enzyme 2 (ACE2) activity, but there is little information about changes in ACE2 in chronic kidney disease (CKD) patients without history of CV disease. We examined circulating ACE2 activity in CKD patients at stages 3–5 (CKD3-5) and in dialysis (CKD5D) without any history of CV disease.

Methods. Circulating ACE2 activity was measured in human ethylenediamine-tetraacetic acid (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 and hypertension; glycaemic, renal, lipid and anaemia profiles; vitamin D analogues treatment and antihypertensive treatments (angiotensin-converting enzyme inhibitor and angiotensin receptor blockade) were analysed. 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, significant decrease in circulating ACE2 activity was observed when compared with 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. Significant increase in the activity of circulating ACE was found in CKD3-5 and CKD5D when compared with CONT and in CKD5D when compared with 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 activities 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.
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 death (death, CV events and hospitalization). This risk is evident at an estimated GFR of <60 mL/min/1.73 m² and increases with an estimated GFR of <45 mL/min/1.73 m² [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 remodelling, 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 increases in heart failure patients, acute myocardial infarction [14], and correlates with the severity of the heart disease [15]. Circulating ACE2 levels 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 haemodialysis 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 and ACE 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.

MATERIALS AND METHODS

Patients and variables

A total of 2572 subjects from an observational and multicentre 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, ischaemic stroke, haemorrhagic stroke, abdominal aortic aneurysm and atherosclerosis) and in the age range between 18 and 74 years were included in the study. Exclusion criteria were pregnancy, human immunodeficiency virus infection, any type of transplantation or history of transplantation, previous history of carotid artery disease, patients with active infections and/or hospitalized in the last month, and intercurrent illness that presumes the absence of follow-up or survival expectation less than 1 year.

Patients were classified into three groups according to their GFR [modification of diet in renal disease (MDRD)-4]: 1458 non-dialysis patients with CKD at stages 3–5 (CKD3-5, MDRD-4 <60 mL/min/1.73 m²), 546 in dialysis (haemodialysis or peritoneal dialysis) patients (CKD5D) and 568 subjects with MDRD-4 ≥60 mL/min/1.73 m² were used as controls (CONT).

Clinical variables analysed were gender, age, history of diabetes, hypertension, dyslipidemia and smoking (active smokers over the last month). Angiotensin-converting enzyme inhibitor (ACEi), angiotensin receptor blockade (ARB), diabetes medication (insulin and oral antidiabetic drugs) and vitamin D analogues treatments were recorded. Analytical variables analysed were glycaemic (blood glucose and glycosylated haemoglobin), lipid and anaemia profiles. The presence of plaques was determined by ultrasound of the carotid arteries.

Ethylendiamine-tetraacetic acid (EDTA)-anticoagulated plasma samples were collected from all patients and controls, centrifuged at 3000 g 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 in 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 EDTA, a chelating agent of loosely bound metal ions such as Zn²⁺, which acts as a cofactor for carboxypeptidases. As EDTA inhibits ACE2 and ACE activities [22], zinc chloride (ZnCl₂) was added to the plasma samples to avoid its binding. Briefly, 2 µL of plasma was incubated with buffer (100 mM Tris-HCl, 600 mM NaCl, 10 µM ZnCl₂, pH 7.5) in the presence of protease inhibitors (100 µM captopril, 5 µM amastatin, 5 µM bestatin and 10 µM Z-Pro-linal). Plasma samples were collected into tubes containing EDTA, a chelating agent of loosely bound metal ions such as Zn²⁺, which acts as a cofactor for carboxypeptidases. As EDTA inhibits ACE2 and ACE activities [22], zinc chloride (ZnCl₂) was added to the plasma samples to avoid its binding. Briefly, 2 µL of plasma was incubated with buffer (100 mM Tris-HCl, 600 mM NaCl, 10 µM ZnCl₂, pH 7.5) in the presence of protease inhibitors (100 µM captopril, 5 µM amastatin, 5 µM bestatin and 10 µM Z-Pro-linal). Plasma samples were collected into tubes containing EDTA, a chelating agent of loosely bound metal ions such as Zn²⁺, which acts as a cofactor for carboxypeptidases. As EDTA inhibits ACE2 and ACE activities [22], zinc chloride (ZnCl₂) was added to the plasma samples to avoid its binding. Briefly, 2 µL of plasma was incubated with buffer (100 mM Tris-HCl, 600 mM NaCl, 10 µM ZnCl₂, pH 7.5) in the presence of protease inhibitors (100 µM captopril, 5 µM amastatin, 5 µM bestatin and 10 µM Z-Pro-linal).
reaction buffer (final volume 100 µL) at 37°C for 16 h. The optimal concentration of ZnCl₂ for the determination of ACE2 activity was 0.5 mM. 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 recombinant human ACE2 (rhACE2) adding increasing quantities of rhACE2 (0, 1, 2, 4, 8 and 16 ng) (Figure 1C). In a set of experiments, serum and EDTA-plasma samples were incubated with rhACE2 in the presence or absence of ZnCl₂. 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]. ZnCl₂ was tested to find the optimal concentration for reversing the effect of EDTA.

Briefly, 0.83 µL of plasma with different concentrations of ZnCl₂ (0, 7.81, 15.63 and 31.25 mM) (Figure 1B) was incubated with 73 µL of appropriate buffer (0.5 M borate buffer and 5.45 M N-hippuryl-His-Leu (HHL)) at 37°C for 25 min. Finally, the fluorescent adduct of the enzyme-catalysed product L-histidyl-L-leucine was quantified. A concentration of 15.63 mM 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 continuous variables was assessed by normal probability plots. Variables were expressed as mean ± SE. Paired case-control studies were performed: CONT versus...
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 CV disease, divided into those not requiring dialysis (CKD3-5) and those in dialysis (CKD5D). Characteristics of study subjects are shown in Table 1. CKD5D patients 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 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, and 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 ZnCl2 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 ZnCl2 was added to the EDTA-plasma with rhACE2, ACE2 activity was detected. These results demonstrated that within EDTA-plasma neither endogenous nor 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 significantly decreased in CKD3-5 as compared with CONT (45.4 ± 1.12 versus 52.9 ± 1.50, P < 0.001) and in CKD5D as compared with CONT.

Table 1. Clinical characteristics of study cohorts

| Age (years) | Total population | CONT | CKD3-5 | CKD5D | P-value |
|-------------|------------------|------|--------|--------|---------|
| Male/female | 1555/1017        | 347/272 | 1093/662 | 409/279 | P = 0.020 |
| Diabetes    | 584 (22.7%)      | 76 (12.3%) | 506 (28.8%) | 124 (18.0%) | P < 0.001 |
| Hypertension| 2006 (78%)       | 248 (40.1%) | 1588 (90.5%) | 590 (85.8%) | P < 0.001 |
| Dyslipidemia| 1491 (58%)       | 231 (37.3%) | 1215 (69.2%) | 363 (52.8%) | P < 0.001 |

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, angiotensin-converting enzyme inhibitors; ARB, angiotensin II receptor blockers.

Table 2. ICCs and percentage of recovery for ACE2 and ACE activities between different sample conditions

| ICC (95% Confidence interval) | ACE2 activity | ACE activity |
|------------------------------|--------------|--------------|
| EDTA-plasma ZnCl2 sample versus serum sample | 0.95 (0.91–0.97) | 0.90 (0.90–0.95) |
| Serum sample versus plasma-heparin sample | 0.95 (0.91–0.98) | 0.93 (0.86–0.97) |
| EDTA-plasma ZnCl2 sample versus plasma-heparin sample | 0.97 (0.94–0.98) | 0.84 (0.68–0.92) |

% Recovery

| EDTA-plasma ZnCl2 sample versus serum sample | 107% | 89% |
| EDTA-plasma ZnCl2 sample versus plasma-heparin sample | 91%  | 83% |
(38.5 ± 1.62 versus 52.9 ± 1.50, P < 0.001). In addition, ACE2 significantly decreased in CKD5D as compared with 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 analysed all groups separately.

Males had significantly increased ACE2 activity when compared with females in all studied groups (P < 0.001). Furthermore, patients with plaques also had increased ACE2 activity as compared with those without plaques (P < 0.001) (Table 3). CONT and CKD3-5 with diabetes showed increased circulating ACE2 activity as compared with 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, smokers had significantly increased circulating ACE2 activity as compared with non-smoker CKD3-5 (P = 0.03) (Table 3).

We found a significant direct correlation between ACE2, age and glycosylated haemoglobin 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 significantly increased in CONT and CKD5D on ARBs therapy as compared with non-treated patients (P = 0.002). Treatment with ACEi had no influence on circulating ACE2 (Table 3). ACE2 activity also increased in CONT (P = 0.007) and CKD3-5 (P < 0.001) under oral anti-diabetic agents as compared with non-treated. In addition, insulin therapy increased ACE2 activity in CKD3-5 (P < 0.001) (Table 3). Surprisingly, circulating ACE2 decreased in CKD5D treated with cholecalciferol as compared with 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 significantly increased in CKD3-5 as compared with CONT (4181.65 ± 58.37 versus 3809.13 ± 71.96, P = 0.035) and in CKD5D as compared with CONT (4454.48 ± 87.10 versus 3809.13 ± 71.96, P < 0.001). In addition, ACE activity significantly increased in CKD5D as compared with 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, the same results were observed (Figure 3B).

Females had increased circulating ACE activity as compared with males in CONT and CKD3-5 (P < 0.001). However, no differences were observed in CKD5D (P = 0.057) (Table 3). CONT with the presence of plaques showed decreased levels of circulating ACE activity as compared with those without plaques (P = 0.011). However, no differences were observed in CKD3-5 and CKD5D. ACE activity decreased in CKD3-5 with hypertension or dyslipidemia as compared with 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 haemoglobin (P = 0.019) in CONT.

**FIGURE 2:** Circulating ACE2 activity between studied groups. (A) ACE2 activity significantly decreased in CKD3-5 (grey bars) and CKD5D (white bars) patients as compared with CONT (black bars) (*P < 0.001). CKD3-5 showed an increase in plasma ACE2 activity as compared with CKD5D (**P < 0.001). When matching samples with an equal distribution of gender, diabetes, hypertension, dyslipidemia, smoking habits, weight and age (B), ACE2 activity significantly decreased in CKD3-5 and CKD5D as compared with 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.
Increased ACE activity was found in CONT and CKD3-5 in ARBs therapy as compared with non-treated patients. As expected, subjects treated with ACEi had lower levels of ACE activity as compared with non-treated subjects in all groups. ACE activity decreased in CKD3-5 and CKD5D treated with cholecalciferol as compared with 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 circulating ACE activity in CONT.

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

| Clinical variables | Circulating ACE2 activity (RFU/µL/h) ± SEM | Circulating ACE activity (RFU/µL) ± SEM |
|--------------------|-------------------------------------------|----------------------------------------|
|                    | 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*   | 27.2 ± 1.37* | 4144.1 ± 106.9*| 4426.9 ± 96.8* | 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*   | 44.0 ± 6.06  | 4217.7 ± 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*   | 56.6 ± 3.27    | 41.8 ± 2.19* | 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         |                |                |              |                |                |              |
| 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 ± 1.59    | 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, angiotensin-converting enzyme inhibitors; ARB, angiotensin II receptor blockers.

*P < 0.05 (no versus yes, male versus female and absence versus presence).

### 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 decreased in CKD3-5 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...
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 Chinese hamster ovary 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 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 study groups. Note that in our study, patients had no history of CV disease. In agreement with Roberts et al., we initially 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 haemodialysis 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 CONTROL, 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

| Table 4. Multiple linear regression analysis of potential predictors of circulating ACE2 and ACE activities |
|-------------------------------------------------|
| Predictors of circulating ACE2 activity          |
| (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| 0.118  |
| Predictors of circulating ACE Activity          |
| (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-value. 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, angiotensin-converting enzyme inhibitors; ARB, angiotensin II receptor blockers.

**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 with CONT (black bars). CKD5D showed an increase in plasma ACE activity as compared with 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.
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 tumour necrosis factor-α convertase ADAM17 [27–29]. It is possible that the increase in ACE2 in males may be related to the increase in ADAM17 shedding. Further studies focused on 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 infract size [14]. Furthermore, KT patients with a previous history of ischaemic heart disease presented increased ACE2 activity [17]. Soro-Paavonen et al. demonstrated that circulating ACE2 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 also increases in diabetic CKD patients and it correlates with glycosylated haemoglobin. In mice with experimental diabetes, ACE2 activity 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 increase starts 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 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 a 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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CONFLICT OF INTEREST STATEMENT

None declared.

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Background. Anti-neutrophil cytoplasm antibody (ANCA) associated vasculitis with renal involvement requires treatment with potentially toxic drugs to reduce morbidity and mortality, and there is a major challenge to determine clinical and histological features predictive of renal prognosis. The aim of our study was to evaluate the use of the 2010 international histological classification for ANCA-associated glomerulonephritis (AAGN) as a predictor of renal outcome when used in conjunction with other prognostic factors.

Methods. One hundred and four patients with AAGN treated at our centre were included: 23 were classified as focal, 26 as crescentic, 48 as mixed and 7 as sclerotic. Renal outcomes were based on estimated glomerular filtration rate (eGFR) at 1 and 5 years, and on renal survival.

Results. By univariate analysis, patients in the focal class had the best renal outcome, those in the sclerotic class the worst outcome, and those in the mixed and crescentic classes had intermediate renal survival. There was no significant difference in outcome between the mixed and crescentic classes. In multivariate models, histological class did not improve model fit or associate with renal outcome after adjusting for established prognostic factors. Lower percentage of normal glomeruli, greater degree of tubular atrophy (TA), MPO-ANCA positivity, increasing age and lower starting eGFR, all correlated with poorer renal outcomes.

Conclusions. We conclude that, in our cohort of patients, the international histological classification is predictive of renal outcome in AAGN, but did not appear to be additionally informative over other established prognostic factors in multivariate analysis. However, it may be of value to combine the current histological classification with other established parameters, such as TA and percentage normal glomeruli.

Keywords: ANCA, clinical outcome, glomerulonephritis, renal pathology, vasculitis