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CIRCULATING ACE2 AND KIDNEY DISEASE: MODULATION OF EXPRESSION AND VALUE AS A BIOMARKER OF ATHEROMATOSIS PROGRESSION

Thesis submitted by

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PARTICIPATION IN CONGRESSES

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INTERNATIONAL CONGRESSES
Oral Communications
- **L Anguiano**, M Riera, J Pascual, C Barrios, A Betriu, JM Valdivielso, E Fernández, MJ Soler. *Circulating angiotensin-converting enzyme 2 activity as a biomarker of atherosclerosis progression in patients with chronic kidney disease.* 50th European Renal Association-European Dialysis and Transplantation Congress. Istanbul, May 2013.

Posters
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- **L Anguiano**, M Riera, J Pascual, C Barrios, A Betriu, JM Valdivielso, E Fernández, MJ Soler. *Circulating ACE and ACE2 in patients with chronic kidney disease without history of cardiovascular disease.* 51th European Renal Association-European Dialysis and Transplantation Congress. Amsterdam, May 2014.
- M Riera, **L Anguiano**, M Rebull, S Clotet, J Pascual, MJ Soler. *Paricalcitol modulates plasma ACE2 activity and renal ADAM17 content in type 1 diabetic mice.* 47th American Society of Nephrology Congress. Philadelphia, November 2014.
- **L Anguiano**, M Riera, J Pascual, C Barrios, A Betriu, JM Valdivielso, E Fernández, MJ Soler. *Circulating ACE2 as a biomarker of chronic kidney disease.* 48th American Society of Nephrology Congress. Chicago, November 2015.

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- M Riera, **L Anguiano**, S Clotet, J Pascual, MJ Soler. *Paricalcitol modula la actividad circulante del enzima conversor de angiotensina 2 (ECA2) y ADAM17 renal en un modelo de ratón de diabetes tipo 1.* XLIV Congreso Sociedad Española de Nefrología. Barcelona, October 2014.

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ABBREVIATIONS

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ABI	Ankle-brachial index	**DNA**	Deoxyribonucleic acid
ACE	Angiotensin-converting enzyme	**EDTA**	Ethylenediamine-tetraacetic acid
ACE2	Angiotensin-converting enzyme 2	**ESRD**	End-stage renal disease
ACR	Albumin-to-creatinine ratio	**GBM**	Glomerular basement membrane
AD	Atheromatous disease	**GFR**	Glomerular filtration rate
ADAM17	A desintegrin and metalloproteinase domain 17	**H_2O_2**	Hydrogen peroxide
		IMT	Intima-media thickness
AER	Albumin excretion rate	**KO**	Knockout
AGE	Advanced glycation end products	**Ln**	Natural logarithm
		M	Molar
Agt	Angiotensinogen	**MDRD**	Modification of diet in renal disease
AKI	Acute kidney injury		
Ang 1-7	Angiotensin 1-7	**mg**	Milligrams
Ang I	Angiotensin I	**MGV**	Mean grey value
Ang II	Angiotensin II	**mL**	Milliliter
ARB	Angiotensin receptor 1 blockers	**mM**	Millimolar
		mmHg	Millimeter of mercury
AT_1 receptor	Angiotensin 1 receptor	**MTC**	Mouse tubular cells
AT_2 receptor	Angiotensin 2 receptor	**N-terminal**	Amino-terminal
AU	Arbitrary units	**NADPH**	Nicotinamide adenine dinucleotide phosphate
bpm	Beats per minute		
C-terminal	Carboxi-terminal	**nm**	Nanometers
CHO	Chinese hamster ovary cells	**NOD**	Nonobese diabetic
		NOR	Nonobese resistant
CKD	Chronic kidney disease	**PCR**	Polymerase chain reaction
CV	Cardiovascular		
Da	Dalton	**pg**	Picograms
DBP	Diastolic blood pressure	**PKC**	Protein kinase C
DN	Diabetic nephropathy	**RAS**	Renin-angiotensin system

RFU	Relative fluorescence units	**TGF**	Transforming growth factor
ROS	Reactive oxidative stress	**TNF**	Tumor necrosis factor
SBP	Systolic blood pressure	**UAE**	Urinary albumin excretion
SD	Standard deviation	**VDR**	Vitamin D receptor
SE	Standard error	**μg**	Micrograms
STZ	Streptozotocin	**μL**	Microliters
TACE	Tumor necrosis factor-α converting enzyme	**μM**	Micromolar

SUMMARY

SUMMARY

Angiotensin-converting enzyme (ACE) 2 is a carboxypeptidase that degrades angiotensin (Ang) II to Ang 1-7, thus counteracting the vasoconstrictor and deleterious effects of Ang II. Circulating ACE2 activity is increased in patients with cardiovascular (CV) disease and in experimental models of diabetic nephropathy (DN). Renin-angiotensin system (RAS) blockade by ACE inhibitors and angiotensin II type 1 receptor blockers (ARBs) have been considered the gold standard treatment in patients with kidney disease. However, there is a need for new therapeutic strategies to slow-down the progression of kidney disease. In this regard, the vitamin D analog paricalcitol has been suggested as a novel therapeutic agent to protect against DN. Given these premises, two studies have been proposed in this thesis: a) to evaluate the role of circulating ACE2 as a biomarker of CV disease in chronic kidney disease (CKD) patients, and b) to study the role of paricalcitol in modulating ACE2 in a mouse model of DN. For the human study, baseline circulating ACE2 activity was measured in human plasma samples from the NEFRONA Study, which includes CKD patients without previous history of CV disease. Baseline and prospective studies were performed to evaluate the association of circulating ACE2 with baseline clinical and analytical variables, and with silent atherosclerosis and CV outcomes during the 2-year and 4-year follow-up. For the experimental study, female non-obese diabetic (NOD) mice were studied after diabetes onset and divided into different treatment groups: low-dose and high-dose paricalcitol, aliskiren and a combination of paricalcitol and aliskiren. The effect of paricalcitol was also studied in proximal tubular epithelial cells. The human study showed: 1) CV risk factors, such as male gender, older age and diabetes were found as independent predictors of ACE2 activity in CKD patients; 2) higher number of territories with plaques at 2 years of follow-up was independently associated with higher levels of baseline circulating ACE2 activity in CKD patients; and 3) cox regression analysis confirmed an association between baseline circulating ACE2 activity and CV and non-CV outcomes at 4 years of follow-up in CKD patients. Within the experimental study: 1) paricalcitol alone or in combination with aliskiren resulted in significantly reduced circulating ACE2 activity in NOD mice, beyond the glycemic profile; 2) there were no significant changes in urinary albumin excretion; 3) renal content of ADAM17 was significantly decreased by treatment with high-dose paricalcitol; 4) renal and circulating oxidative stress were reduced in high-dose paricalcitol-treated mice; and 5) in culture, paricalcitol incubation resulted in a significant increase in ACE2 expression compared with untreated cells. In summary, ACE2 activity can be modulated by administration of paricalcitol, counterbalancing the effect of diabetes on circulating ACE2 activity. Paricalcitol may modulate circulating ACE2 by reducing renal ADAM17 content, thus blocking ACE2 shedding from the membrane. In CKD patients without previous history of CV disease, circulating ACE2 activity can also serve as a biomarker of silent atherosclerosis and CV outcomes. Therefore, clinical assessment of circulating ACE2 levels and ACE2 modulators may be beneficial in treating patients with kidney disease.

I. INTRODUCTION

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A. *Chronic Kidney Disease*

A.I. Definition and classification

Kidney disease is an abnormality of kidney structure or function with implications for the health of an individual, which can occur abruptly, and either resolve or become chronic. Chronic kidney disease (CKD) is defined as an alteration in kidney structure and function with variable clinical presentation, depending on the cause, severity and rate of progression of the disease. These abnormalities must have implications for health and be present for more than 3 months. KDOQI clinical practice guidelines established two criteria for defining CKD [1,2] (Table 1).

Table 1. **Criteria for definition of CKD.**

Criteria for definition of CKD
Kidney damage for more than 3 months, with or without decreased glomerular filtration rate (GFR), manifested by pathological abnormalities or markers of kidney damage
GFR < 60 mL/min/1.73m^2 for more than 3 months, with or without kidney damage

Kidney damage can be displayed within the parenchyma, large blood vessels or collecting systems, and can be detected by several markers rather than direct examination of kidney tissue. Markers of kidney damage include:
- Proteinuria, defined as increased amounts of protein in urine. Proteinuria reflects abnormal loss of plasma proteins due to several factors, such as increased glomerular permeability to large molecular weight proteins, incomplete tubular reabsorption of normally filtered low molecular weight proteins, or increased plasma concentration of low molecular weight proteins. Experimental and clinical studies have suggested an important role of proteinuria in the pathogenesis of disease progression of CKD [3].
- Albuminuria, which refers to abnormal loss of albumin in the urine. Albumin is one type of plasma protein found in the urine in normal subjects and in larger quantity in patients with kidney disease. Albuminuria in CKD is defined as an albumin excretion rate (AER) ≥ 30 mg/24 hours, or albumin creatinine ratio (ACR) ≥ 30 mg/g. An AER ≥ 30 mg/24 hours is greater than 3 times the normal value in young adult men and women of approximately 10 mg/24 hours (ACR 10 mg/g) and is associated with higher risk of complications, such as, all-cause and cardiovascular (CV) mortality, kidney failure, acute kidney injury (AKI) and CKD progression [4,5].
- Urine sediment abnormalities.
- Electrolyte and other abnormalities due to tubular disorders, which may result from disorders of renal tubular reabsorption and secretion.

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- Pathological abnormalities directly observed in kidney tissue obtained by biopsy.
- Structural abnormalities detected by imaging.
- History of kidney transplantation.

The GFR is widely accepted as the best overall index of kidney function because it is reduced after structural damage. A GFR < 60 mL/min/1.73m^2 is less than half of the normal value in young adult men (130 mL/min/1.73m^2) and women (120 mL/min/1.73m^2), and is associated with a higher risk of complications of CKD than in subjects with stable GFR and CKD [6,7].

CKD is classified based on cause, GFR category (Table 2), and albuminuria category (Table 3) [2].

Table 2. **GFR categories in CKD.**

GFR category	GFR (mL/min/1.73m^2)	Terms
G1*	≥ 90	Normal or high
G2*	60-89	Mildly decreased
G3a	45-59	Mildly to moderately decreased
G3b	30-44	Moderately to severely decreased
G4	15-29	Severely decreased
G5	< 15	Kidney failure

*In the absence of evidence of kidney damage, neither GFR category G1 nor G2 do not constitute CKD.

Table 3. Albuminuria categories in CKD.

Albuminuria category	AER (mg/24 hours)	ACR (mg/g)	Terms
A1	< 30	< 30	Normal to mildly increased
A2	30-300	30-300	Moderately increased
A3	> 300	> 300	Severely increased

A.II. Evaluation

When CKD is diagnosed, evaluation and treatment of patients with CKD should be performed. For the evaluation of patients with CKD, several concepts should be evaluated: diagnosis or etiology of kidney disease, severity and complications of CKD (assessed by levels of kidney function), risk for loss of kidney function (see section A.III) and risk for CV disease (see section A.IV).

Classification of the type of kidney disease is based on pathology and etiology. In developed countries, age, hypertension, diabetes, increased body-mass index, smoking, and history of established CV disease are associated consistently with CKD [8–10]. The study from Fox et al. confirmed that in a large community-based sample of 2585 participants free of kidney disease at

baseline, a mildly reduced GFR, diabetes, hypertension, obesity and smoking are important risk factors for the development of new-onset kidney disease [8] (Table 4).

Table 4. Baseline and long-term multivariate predictors of developing kidney disease. Adapted from Fox CS et al. 2004.

	Odds Ratio (95% Confidence Interval)	
	Baseline	**Long-term**
Age, per 10-year increment	2.36 (2.00-2.78)	2.18 (1.84-2.58)
Sex (women vs men)	0.89 (0.67-1.18)	0.96 (0.72-1.27)
Baseline GFR (mL/min/1.73m^2)		
<90	3.01(1.98-4.58)	2.95 (1.94-4.49)
90-119	1.84 (1.16-2.93)	1.87 (1.18-2.98)
Body mass index, per SD unit	1.23 (1.08-1.41)	-
Smoking (yes vs no)	1.42 (1.06-1.91)	-
Diabetes (yes vs no)	2.60 (1.44-4.70)	2.38 (1.45-3.92)
Hypertension (yes vs no)	-	1.57 (1.17-2.12)

Abbreviations: GFR, glomerular filtration rate; SD, standard deviation

Importantly, diabetic kidney disease is the largest single cause of kidney failure in the developed world [11–13]. However, a variety of diseases, including other glomerular diseases, vascular, tubulointerstitial, and cystic diseases (often grouped together under the label of non-diabetic kidney diseases) are also common causes of CKD (Figure 1).
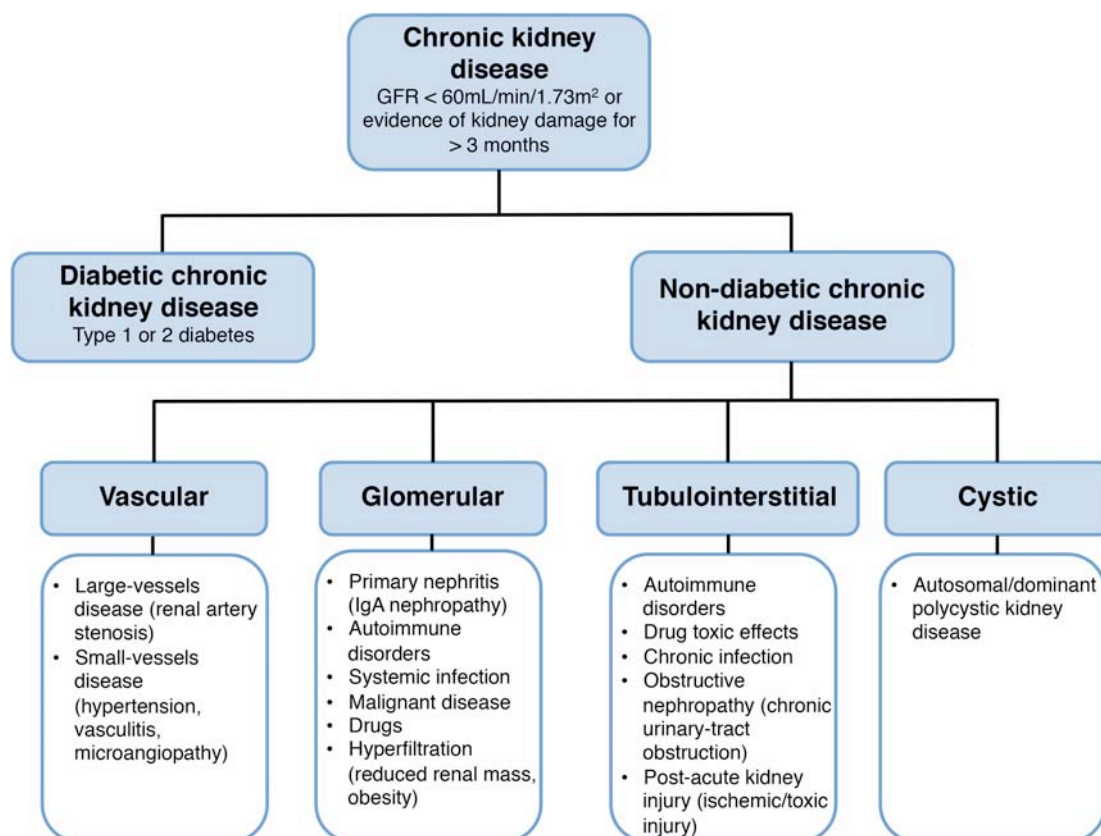
Figure 1. Causes of chronic kidney disease.

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The differential diagnosis of CKD in a specific patient is based on the clinical history, physical examination, and laboratory evaluation. Laboratory measurements in patients with CKD include [1]:

- Serum creatinine.
- ACR in a random untimed urine specimen.
- Examination of the urine sediment for erythrocytes and leukocytes.
- Imaging of the kidneys by ultrasonography.
- Serum electrolytes: sodium, potassium, chloride and bicarbonate.

As it has been mentioned above, GFR is used to measure kidney function. Ideally, it can be measured as the urinary or plasma clearance of an ideal filtration marker such as inulin or of the alternative exogenous markers such as iothalamate, ethylenediaminetetraacetic acid (EDTA), diethylene triamine pentaacetic acid, and iohexol. However, measuring clearance with the use of exogenous markers is complex, expensive and difficult to do in routine clinical practice [14]. Therefore, GFR is estimated with endogenous filtration markers, such as creatinine or cystatin C.

Creatinine is an amino acid derivative with a molecular mass of 113 Da that is freely filtered by the glomerulus and that can be computed from a timed urine collection (24-hour urine collection) and blood sampling during the collection period. Creatinine is generated in muscle from the non-enzymatic conversion of creatine and phosphocreatine. Creatinine is synthesized from arginine and glycine in the liver and actively concentrated in muscle. In the steady state, creatinine generation can be estimated by creatinine excretion and is determined primarily by muscle mass and dietary intake, which probably accounts for the variations in the level of serum creatinine observed among different age, gender or ethnic group [15]. Therefore, the relationship between the levels of serum creatinine and GFR varies substantially among persons and over time.

Numerous equations have been developed to estimate GFR in adults and include variables such as age, sex, race, and body size, in addition to serum creatinine, thus overcoming some of the limitations of the use of serum creatinine alone. The first equation for estimating GFR was the Cockcroft-Gault formula, which was developed in 1973 with data from 249 men with creatinine clearance from 30 to 130 mL/min, and was not adjusted for body-surface area [16]. This equation systematically overestimates GFR because of the tubular secretion of creatinine. In 1999 the Modification of Diet in Renal Disease (MDRD) study equation was developed with the use of data from 1628 patients with CKD. It uses serum creatinine, age, sex and race (black versus white and others) to estimate GFR adjusted for body-surface area (1.73m^2, average value of body-surface are of 25-year old men and women) [17,18]. This equation was re-expressed in 2005 for use with a standardized serum creatinine assay [19]. Concerns have been raised about the use of the MDRD Study equation because of the high prevalence estimates in the

elderly, women and whites, compared to the low incidence rates of treated kidney failure in these groups [20,21]. Thus, the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation was developed in 2009, using the same four variables as the MDRD study equation [22]. This new equation had less bias than the MDRD study equation, especially at GFR ≥60 mL/min/1.73m^2, improving precision and accuracy [22,23].

Cystatin C is a nonglycosylated basic protein with low molecular mass (13 kDa) that is freely filtered by the glomerulus. The generation of cystatin C appears to be less variable from person to person than that of creatinine. Compared to serum creatinine, cystatin C has lower dependency on muscular mass and is more predictable of mortality and end stage renal disease (ESRD) [24,25].

A.III. Prevalence and risk factors for chronic kidney disease

Prevalence of CKD

CKD is a major social health problem and the prevalence of CKD is growing worldwide due to the increase in related diseases as type 2 diabetes, obesity, hypertension or atherosclerosis [9,26].

In a systematic review of more than 25 epidemiological studies, the median prevalence of CKD was 7.2% in people aged 30 years or older and revealed ethnic-specific differences. [27]. In a comparative study between data from the National Health and Nutrition Examination Surveys (NHANES) 1988-1994 with NHANES 1999-2004 the prevalence of CKD and the distribution of CKD stages and severity in the United States (US) population was described [20]. The proportion of the US population with mild, moderate, or severely reduced estimated GFR increased from 1988-1994 to 1999-2004. Overall, the prevalence rate of CKD increased from 10.0% in 1988-1994 to 13.1% in 1999-2004. Moderately reduced GFR increased in prevalence from 5.4% to 7.7%, and the prevalence of severely reduced GFR increased from 0.21% to 0.35% [20]. When analyzing the prevalence estimated for each stage of CKD, it was higher in 1999-2004 than in 1988-1994 with the difference being statistically significant for CKD stages 2 through 4 (Table 5). The overall prevalence of CKD among men was 8.2% in 1988-1994 and 11.1% in 1999-2004, while among women prevalences were 12.2% and 15.0%, respectively. Within age categories, the prevalence of CKD stages 1 and 2 increased from 2% in 1988-1994 to 3% in 1999-2004 at age 20-39 years compared with 9% and 10% after the age of 70 years. The prevalence for stage 3 and 4 combined were 0.2% in 1988-1994 and 0.7% in 1999-2004 at aged 20-39 years compared with 27.8% and 37.8% after the age of 70 years [20].

Within the Spanish population, the Spanish Society of Nephrology initiated a program to identify population at risk for CKD and increase preventive measures in order to reduce the incidence of renal failure, CV complications, and progression to ESRD [28].

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Within this program, the "Estudio Epidemiológico de la Insuficiencia Renal en España" (EPIRCE) was the first epidemiological study at a national level designed to describe the prevalence of CKD in the general Spanish population aged 20 years or older, using the MDRD equation [29]. The overall prevalence of CKD stages 3-5 was 6.83%. When the ACR was added to the diagnostic criteria, the prevalence rose to 9.16%. The prevalence estimates of CKD stages were: 0.99% for stage 1; 1.3% for stage 2; 5.4% for stage 3a; 1.1% for stage 3b; 0.27% for stage 4; and 0.03% for stage 5 (Table 5) [29].

Table 5. Prevalence of CKD Stages in US and Spanish population. Adapted from Coresh J et al. 2007 and Otero A, et al. 2010.

Prevalence of CKD % (95% Confidence Interval)			
	US population		Spanish population
Stage	NHANES 1988-1994 (n=15488)	NHANES 1999-2004 (n=13233)	EPIRCE 2010 (n=2746)
1	1.71 (1.28 to 2.18)	1.78 (1.35 to 2.25)	0.99 (0.57 to 1.4)
2	2.70 (2.17 to 3.24)	3.24 (2.61 to 3.88)	1.3 (0.84 to 1.8)
3 3a	5.42 (4.89 to 5.95)	7.69 (7.02 to 8.36)	5.4 (4.3 to 6.6)
3b			1.1 (0.65 to 0.48)
4	0.21 (0.15 to 0.27)	0.35 (0.25 to 0.45)	0.27 (0.06 to 0.48)
5	-	-	0.03 (0.00 to 0.08)
Total	10.03 (9.16 to 10.91)	13.07 (12.04 to 14.10)	6.83* (5.41 to 8.25)

*Overall prevalence of CKD stages 3-5
Abbreviations: CKD, chronic kidney disease; NHANES, National Health and Nutrition Examination Surveys; EPIRCE, "Estudio Epidemiológico de la Insuficiencia Renal en España"

Risk factors

The increasing prevalence of CKD is partly explained by the increase in the number of risk factors, including population aging and an increase in the proportion of individuals with obesity, diagnosed diabetes, and hypertension [30,31]. Results from the study from Coresh et al., confirmed that an increasing prevalence of diagnosed diabetes and hypertension in US population had contributed to an increase in the CKD prevalence from 1988-1994 to 1999-2004, which may propagate to higher rates of complications and kidney failure requiring dialysis or transplantation [20]. A systematic review of the published data on the prevalence of CKD in population-based studies confirmed that elderly had a markedly higher prevalence of CKD and that it increased with age in all populations, particularly among persons aged 70 years or older. This study suggests that the increase in the prevalence of CKD

in the elderly might be partly due to related comorbidities of CKD, such as CV diseases or diabetes [27]. Furthermore, a gender-different prevalence of CKD was revealed in most of the included studies from the systematic review. Females had higher prevalence of CKD than males. Given that females have less muscle mass as compared to males and that the muscle mass is a major determinant of serum creatinine concentration, the higher prevalence of CKD in females may be due to an inaccurate correction factor for females in MDRD equation [27]. In fact, the register of renal patients from Catalonia have demonstrated a higher prevalence rate of treated ESRD for men than for women [13]. Results from the EPIRCE epidemiological study in the Spanish population confirmed advanced age, obesity and previously diagnosed hypertension as independent predictor factors for CKD [29].

A.IV. Chronic kidney disease and cardiovascular outcomes

CV disease has been described as the leading cause of morbidity and mortality in patients with CKD [32–34]. A longitudinal study aimed to examine the prevalence of comorbid conditions associated with CKD over a 5-year observation period, demonstrated that patients with CKD have a rate of mortality that exceeds the rate of renal replacement therapy. Patients with CKD stage 4 had a 17.6% risk of progression to kidney failure and a 45.7% mortality risk, compared with a 0.07% and 10.2%, respectively, in patients without CKD [35]. In addition, the study by Go et al. in a large, community-based population described that the risks of death from any cause, CV events, and hospitalization increase as the estimated GFR declines. These risks are evident at an estimated GFR of less than 60 mL/min/1.73m^2 and substantially increase with an estimated GFR of less than 45 mL/min/1.73m^2 [33]. Therefore, the role of accelerated atheromatosis has recently been questioned, given that most CV deaths in dialysis patients are due to sudden death and heart failure [36].

Classic risk predictions equations based on the Framingham Risk Score, a gender-specific algorithm aimed to estimate the CV risk of an individual [37], have been demonstrated to underestimate CV disease risk in adults with CKD [38,39], in which most events occur in patient with low-moderate risk [40,41]. Therefore, there is a need to search for new tools to predict risk for renal patients.

In the Spanish population, the National Observatory of Atherosclerosis in Nephrology (NEFRONA) Study was designed as a multicenter prospective observational cohort study to evaluate the subclinical atherosclerosis burden and the predictive value of carotid and/or femoral ultrasound in a group of CKD patients without previous history of CV disease [42–44]. In this population, the presence of atheromatous plaques was higher among CKD patients on dialysis or patients in CKD stage 4-5 than among non-CKD patients [42,43]. Thus, the higher prevalence of atherosclerosis among dialysis patients compared with a population with normal renal function

supports the existence of dialysis-specific risk factors that could increase the risk of having an atheromatous plaque. In addition, the presence of atheromatous plaques was increased with age, which interacted significantly with sex, although this association was weaker for women than for men (Figure 2) [42]. Atheromatous disease was more prevalent among diabetics and male patients and there was a higher prevalence of atheromatous plaques in both sexes and at any stage of CKD in the context of diabetes.
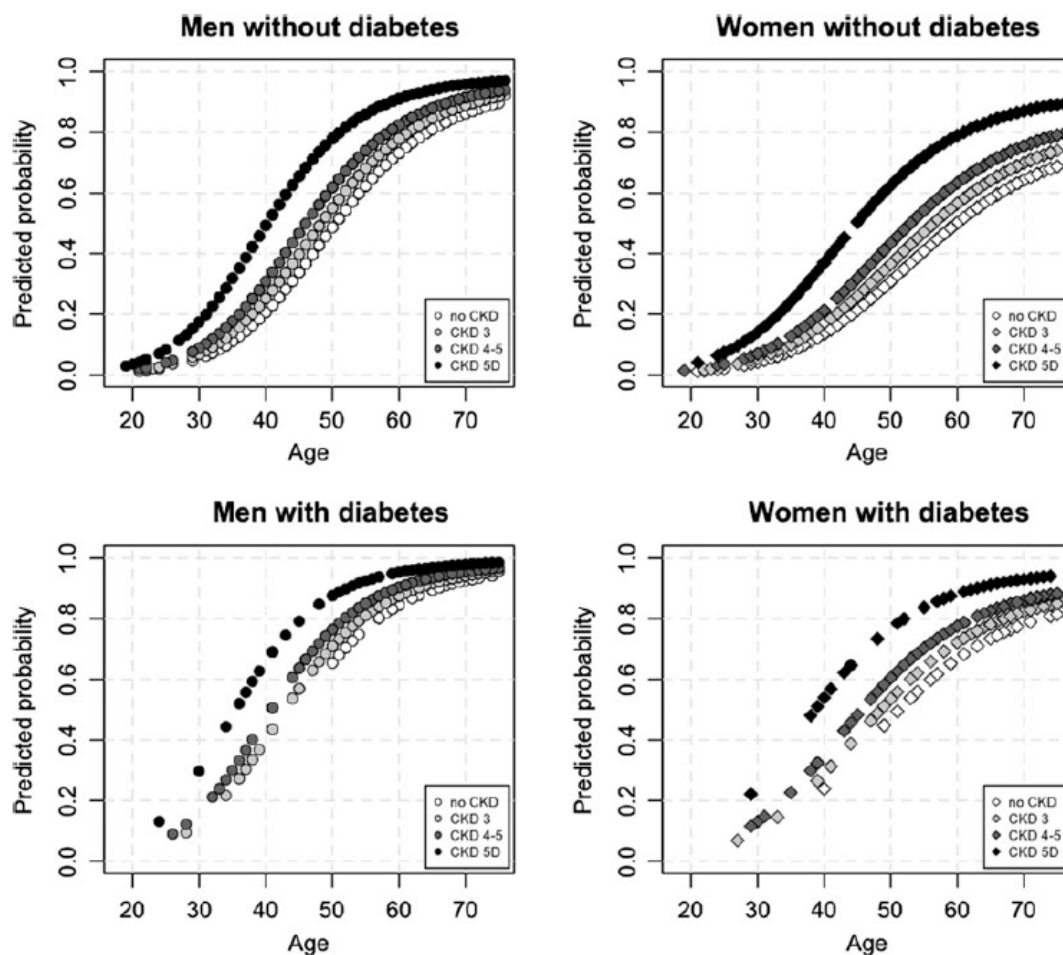
Figure 2. Estimated prevalence of plaque at carotid or femoral arteries including gender, diabetes status, CKD stage and the quadratic effect of age. Figure adapted from Betriu A, et al. 2014.

Baseline data from the NEFRONA Study has also shown that there is a high rate of femoral plaques, even in patients with no carotid atheromatosis [43]. Previous evaluation of this site by radiology, associated femoral plaques with peripheral artery disease and CV prognosis [45]. Thus, some studies have started to explore the evaluation of femoral plaques by ultrasound in general population and in other pathologies [46,47], suggesting that to perform and adequate vascular risk assessment, femoral ultrasound should complement carotid ultrasound.

Results from the progression over 2 years of the NEFRONA Study patients, showed that the percentage of patients with plaque in carotid and/or

femoral territories increased from 68.6% to 81.4% and that atheromatosis progression occurred in 59.8% of patients. It has been demonstrated that CKD is associated with atheromatosis progression, being more frequent in patients with CKD progression and that being diabetic predicts atheromatosis progression independent of the CKD stage [44].

B. Diabetic nephropathy

B.I. Definition

Diabetic nephropathy (DN) is a microvascular complication of type 1 and type 2 diabetes mellitus, that has been classically defined as increased protein excretion in urine [11]. DN is the leading cause of CKD in patients starting renal replacement therapy and is associated with increased CV mortality [21]. DN is a potentially devastating complication of diabetes, and its incidence has more than doubled in the past decade, largely due to the rising prevalence of obesity and type 2 diabetes. It has been estimated that patients with diabetes have a 12-fold increased risk of ESRD compared to patients without diabetes [48].

B.II. Clinical evaluation

Proteinuria and progressive loss of kidney function are the clinical hallmarks of DN. In the early stages of DN there is an increase in the urinary albumin excretion (UAE), defined as an ACR in urine between 30 and 299 mg/g (microalbuminuria) [11]. Subsequently, there is a progressive increase in proteinuria, defined as an ACR ≥ 300 mg/g (macroalbuminuria) and considered as overt DN. A progressive increase in proteinuria leads to a variable decline in renal function (Figure 3). Once the subject has developed macroalbuminuria, the expected GFR decline is 1.2 mL/min/month in type 1 diabetes [49]. In type 2 diabetes, the rate of GFR decline is less predictable, and a mean of approximately 0.5 mL/min/month has been described [50]. However, although measurement of proteinuria is essential to diagnose DN, there are some patients who present decreased GFR when ACR values are normal [51]. Based on this, the classification of the National Kidney Foundation can also be used to stage CKD in these patients [1,2].

Initial studies demonstrated that nearly 80% of patients with microalbuminuria progressed to proteinuria over a period of 6-14 years [52,53]. However, in more recent studies, only 30-45% of microalbuminuric patients progress to proteinuria over 10 years [54]. In fact, several studies have demonstrated a regression in microalbuminuria up to 50-60% of patients, probably associated to a better blood pressure and glycemic control strategies [55,56].
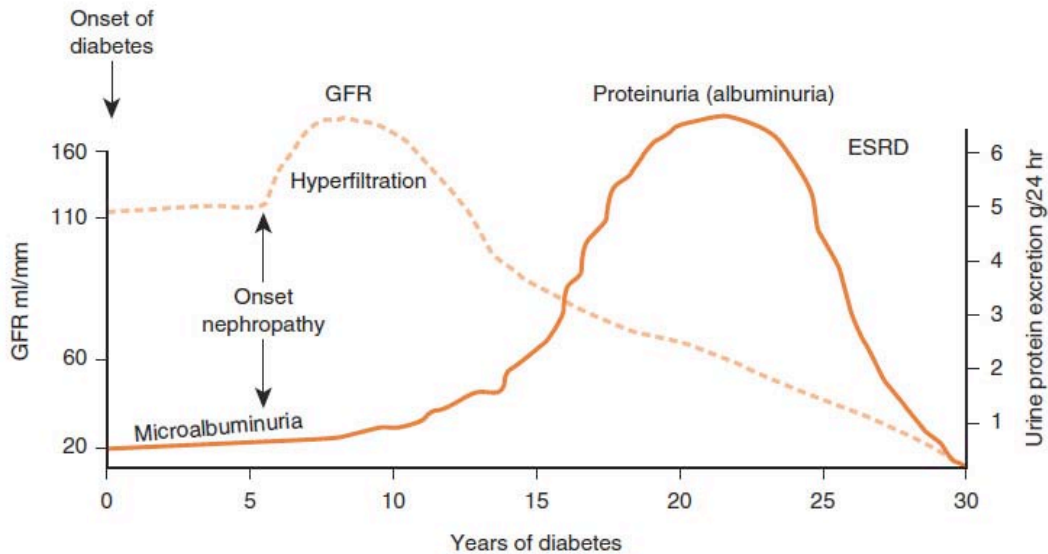
Figure 3. Natural history of DN. Changes in glomerular filtration rate (GFR) and proteinuria. Abbreviations: GFR, glomerular filtration rate; ESRD, end-stage renal disease. Adapted from Williams ME, et al. 2010.

Stages in the development of diabetic nephropathy

Mogensen et al. first characterized the natural evolution of DN into several distinct phases that can be used for both forms of diabetes (Table 6) [57]. Initial changes include glomerular hyperfiltration and hyperperfusion. The second phase is characterized by hyperfiltration and is associated with subtle morphological changes including thickening of the glomerular basement membrane (GBM), glomerular hypertrophy, mesangial expansion, and modest expansion of the tubulointerstitium. This second phase is followed by changes in proteinuria that lead to ACR levels in the range of microalbuminuria (incipient DN). Microalbuminuria has been associated with other microvascular complications, glomerular ultrastructural injury, and endothelial dysfunction or insulin resistance. After the phase of microalbuminuria, there is a continued increase in ACR (macroalbuminuria) with declining GFR and increased blood pressure. Finally, the last phase is characterized by renal failure that will require referral to ESRD programs such as dialysis or transplantation [57,58].

Table 6. Stages in the development of DN. Adapted from Mogensen CE, et al. 1983.

	Designation	Characteristic	GFR (mL/min)	ACR (mg/g)	Blood pressure
Stage 1	Hyperfunction and hypertrophy	Glomerular hyperfiltration	> 150	> 30	Normal
Stage 2	Silent	Thickened GBM and expanded mesangium	~ 150	> 30	Normal
Stage 3	Incipient DN	Microalbuminuria	~ 130	30-299	Increased
Stage 4	Overt DN	Macroalbuminuria	< 100	> 300	Hypertension
Stage 5	Uremic	ESRD	0-10	> 300	Hypertension

Abbreviations: GFR, glomerular filtration rate; ACR: albumin-creatinine ratio; GBM, glomerular basement membrane; DN, diabetic nephropathy; ESRD: end-stage renal disease.

Pathophysiology

In DN there is an interplay of metabolic (glycaemia) and hemodynamic (blood pressure) pathways in the renal microcirculation (Figure 4). Because diabetes is a state of chronic hyperglycemia, it is probable that glucose-dependent processes are involved in DN. On a molecular level, at least five major pathways have been implicated in glucose-mediated vascular and renal damage: (1) increased advanced glycation end-products (AGEs) [59]; (2) increased polyol pathway flux [60]; (3) increased hexosamine pathway flux [61]; (4) activation of protein kinase C (PKC) [62,63]; and (5) stimulation of angiotensin II (Ang II) synthesis [63–65] (Figure 4).

The chronic effects of glucose in inducing tissue injury may occur via the generation of AGEs. AGEs accumulate in the kidney, particularly in people with diabetes and/or declining renal function [59]. Their importance in the pathogenesis of diabetic complications has been demonstrated in animal models, where two structurally unrelated AGEs inhibitors partially prevented various functional and structural manifestations of diabetic microvascular disease [66,67]. Another glucose-dependent pathway, known as the polyol pathway has been implicated in the pathogenesis of DN [60]. This pathway is activated as a response to high levels of intracellular glucose with the objective to metabolize, via the enzyme aldose reductase, glucose to sorbitol. An increased flux of glucose through the hexosamine pathway has also been linked to mechanisms of DN, particularly an increase in transforming growth factor β (TGF-β) [61]. In turn, an increase in TGF-β has been associated with an expansion of the extracellular matrix and increase in the synthesis of fibronectin and collagen [51,63,68]. Intracellular accumulation of glucose also increases *de novo* formation of diacylglycerol, which activates several isoforms of PKC. Finally, glucose-induced Ang II generation is attributed to an activation of the renin-angiotensin system (RAS), via an increase in angiotensinogen expression [65]. Ang II increases TGF-β levels, stimulates

endothelial lesion, increases blood pressure and stimulates cellular proliferation and hypertrophy [63,64]. In addition, at the tissue level, Ang II may be related to the activation of NADPH oxidase, leading to the generation of oxidative stress [64]. As a consequence, presence of Ang II increases tubular reabsorption of proteins and stimulates tissue damage, including proteolysis, inflammation and fibrosis [63].

It has been recently suggested that the central abnormality linking all of these glucose-dependent pathways is oxidative stress, a defect in the mitochondrial electron transport chain resulting in over-production of reactive oxidative stress (ROS) molecules, which stimulate each of the above pathways [69]. An overproduction of ROS activates different signaling pathways, namely MAPK, PI3K/Akt and JAK-STAT. These signaling pathways activate several transcription factors, including NF-kB, AP-1, STAT and EGR1 that have been proposed to promote the synthesis and the secretion of growth factors and cytokines such as TGF-β and IL-6 [70–72]. Activation of TGF-β produces several effects in renal cells (mesangial cells, podocytes, endothelial cells and tubular cells), such as extracellular matrix proteins production, cell proliferation, cell hypertrophy, apoptosis and podocyte abnormalities, among others. These pathophysiological changes of renal cells lead to reduced glomerular filtration and loss of glomerular and interstitial capillaries, tubulointerstitial fibrosis and tubular atrophy, resulting in permanent renal dysfunction [68,73,74] (Figure 4).

Figure 4. Schematic representation of the elements involved in the pathophysiology of DN. Abbreviations: AGE, advanced glycated proteins; ROS, reactive oxygen species; Ang II, angiotensin II.

Histology

The morphologic lesions in DN predominantly affect the glomeruli, with thickening of GBM and mesangial expansion [75]. GBM thickening has been detected as early as 1.5 to 2.5 years after the onset of diabetes. Mesangial expansion, predominantly due to an increase in mesangial matrix, develops later although an increase in the matrix component of the mesangium can be detected as early as 5 to 7 years after the onset of diabetes. Diffuse mesangial expansion can be associated with nodular lesions consisting of areas of marked mesangial expansion forming large round fibrillar mesangial zones with palisading of mesangial nuclei around the periphery of the nodule and compression of the associated glomerular capillaries (Kimmelstiel-Wilson nodules) [75,76]. Both GBM thickening and mesangial expansion are a consequence of extracellular matrix accumulation, with increased deposition of the normal extracellular matrix local components of types IV and VI collagen, laminin and fibronectin [75,77,78]. Tervaert et al. developed a uniform classification system containing specific categories of the main structural glomerular changes and described four classes of glomerular lesions in DN (Table 7) [79].

Table 7. Glomerular classification of DN. Adapted from Tervaert TW et al. 2010.

Class	Description
I	Mild or nonspecific LM changes and EM-proven GBM thickening
IIa	Mild mesangial expansion
IIb	Severe mesangial expansion
III	Nodular sclerosis (Kimmelstiel-Wilson lesion)
IV	Advanced diabetic glomerulosclerosis

Abbreviations: LM, light microscopy; EM, electron microscope; GBM, glomerular basement membrane.

In addition to glomerular lesions, it has also been described a thickening of tubular basement membrane, tubular atrophy and interstitial fibrosis. Vascular lesions can also be detected few years after the onset of diabetes and are characterized by afferent and efferent arteriolar hyalinosis, and a replacement of the smooth muscle cells by plasma proteins, especially immunoglobulins, complement, fibrinogen and albumin (Figure 5) [75].
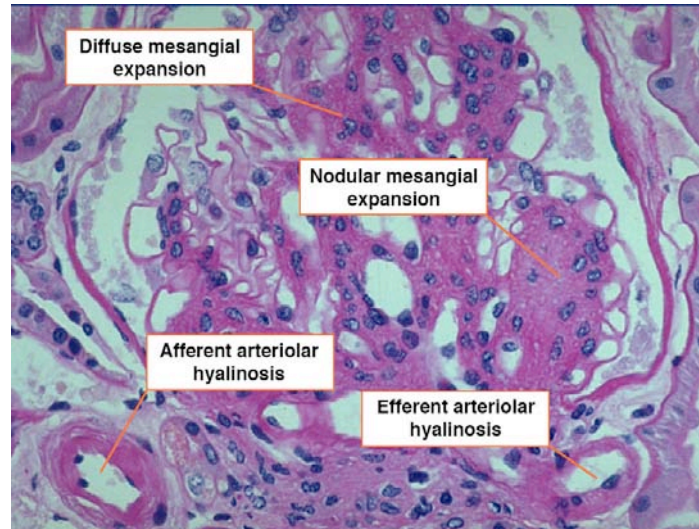
Figure 5. Characteristic lesions of DN. Adapted from Fioretto, P et al. 2007.

C. Animal models of diabetic nephropathy

Biomedical experimentation in mice affords significant advantages over experimentation in other species. These advantages include the development of diverse and unique genetic resources, availability of murine embryonic stem cells, ability to disrupt the expression and function of specific genes, and assembly of repositories of mice that bear multiple mutations [80].

Several studies have been focused in developing animal models to study evolution of DN and new therapeutic strategies. However, there are few animal models that mimic human DN, due to the absence of renal failure. Whereas they exhibit albuminuria, development of glomerular hyperfiltration and some of the characteristic histopathological changes, renal failure from diabetes has not been reported [80,81].

C.I. Validation criteria

The Diabetic Complications Consortium (www.diacomp.org) Nephropathy committee has developed a series of criteria to evaluate the mouse models as they relate to DN disease. These criteria define which measurements must be taken in order to assess this phenotype (Table 8).

Table 8. Functional and structural validation criteria in murine models of DN.

Renal function	
GFR	> 50% reduction over the lifetime of the animal
Albuminuria	Greater than 10-fold increase compared with controls for that strain at the same age and gender
Renal pathology	
Glomerular pathology	Advanced mesangial matrix expansion ± nodular sclerosis and mesangiolysis GBM thickening by > 50% over baseline
Vascular pathology	Any degree of arteriolar hyalinosis
Tubulointerstitial pathology	Tubulointerstitial fibrosis

Abbreviations: GFR, glomerular filtration rate.

Although an ideal model of DN would display all of these criteria, no current model meets them all. Therefore, the criteria should be viewed as goals rather than requirements, and validation of any animal model should include reasonable efforts to exclude other types of kidney disease or damage unrelated to that from diabetes [82].

C.II. Murine models of diabetic nephropathy

The murine models of DN are classified depending on the type of diabetes (type 1 or 2).

The most common murine models of type 1 DN and their phenotypic alterations and kidney pathology are summarized in Table 9. Streptozotocin (STZ)-induced type 1 diabetes has been widely used as a model of DN. However, interpreting results in this model may be complicated by non-specific toxicity of STZ. STZ is a glucosamine-nitrosourea compound presumed to be especially toxic for pancreatic β cells because its glucose moiety is avidly transported into β cells. There, it modifies DNA fragments due to its alkylating properties. DNA damage causes the activation of poly ADP-ribosylation that leads to depletion of cellular NAD+ and ATP. As a result, superoxide radicals are formed, and ROS and a simultaneous cytosolic calcium overload leads to acute necrosis of pancreatic β cells [83]. Depending on the mouse strain, the STZ model develops histologic features described in human DN, such as glomerular hypertrophy, mesangial expansion, arteriolar hyalinosis or nodular glomerulosclerosis [80]. Generally, mice that receive low-dose of STZ develop parallel levels of hyperglycemia to those that receive high-dose STZ. By contrast, the levels of albuminuria are generally lower as a result of reduced direct nephrotoxicity of STZ [80,84].

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Contrary to the STZ-induced model, the non-obese diabetic (NOD) mouse is a spontaneous murine model of type 1 diabetes that has been extensively studied for diabetes. NOD mice develop spontaneous insulinitis at the age of 4-5 weeks, and overt diabetes emerges at the age of 24-30 weeks when most of pancreatic β cells are destroyed. There is a marked sex difference in the incidence of diabetic symptoms in the NOD mouse: female incidence of diabetes is four times higher than in male [85]. The characteristics of autoimmune disease contributing to pancreatic β cell failure have been widely studied and the model has a number of similarities with features of human type 1 diabetes: inheritance of specific major histocompatibility complex (MHC) class II alleles, transmission of the disease by hematopoietic stem cells, and development of an intraislet inflammatory infiltrate (insulinitis) [80]. Studies of DN in NOD mice indicate that albuminuria develops in hyperglycemic NOD animals and that the levels of albuminuria are 7-fold higher than in NOD mice before development of hyperglycemia [86]. Renal lesions show glomerular hypertrophy, structural alteration of the proximal straight tubules, and mesangial proliferation [87,88].

Table 9. Murine models of type 1 DN.

Model	Mechanism	Phenotypic alterations	Kidney pathology
STZ	Acute necrosis of pancreatic β cells	Hyperglycemia Albuminuria Polydipsia Polyuria	Glomerular hypertrophy Mesangial expansion Arteriolar hyalinosis Nodular glomerulosclerosis
NOD	Autoimmune disease contributing to pancreatic β cells failure	Hyperglycemia Albuminuria Polydipsia Polyuria	Glomerular hypertrophy Structural alteration of proximal tubules Mesangial expansion
Insulin-2 Akita	Spontaneous mutation in Ins-2 gene, that leads to the misfolding of insulin protein (toxic to pancreatic β cells)	Hyperglycemia Albuminuria Polydipsia Polyuria	Glomerular mesangial expansion GBM thickening
OVE26	Overexpression of calmodulin that leads to specific damage to pancreatic β cells	Hyperglycemia Albuminuria Polydipsia Polyuria Decline in GFR Hypertension	Diffuse and nodular mesangial expansion Tubulointerstitial fibrosis GBM thickening Glomerular hypertrophy

Abbreviations: STZ, streptozotocin; NOD, nonobese diabetic; GFR, glomerular filtration rate; GBM, glomerular basement membrane.

The common feature in murine models of type 2 diabetes that develop DN is the development of obesity and insulin resistance. The mouse models of type 2 diabetes studied for DN are summarized in Table 10.

The LepR^{db}/LepR^{db} (db/db) mouse is currently the most widely used mouse for studying DN in settings of type 2 diabetes. The db/db mouse has a G-to-T mutation in the gene coding the leptin receptor, responsible for regulating sensation of satiety [89]. Thus, mice develop obesity, insulin resistance, and type 2 diabetes spontaneously. DN in the db/db mouse is initially expressed as increased UAE at the age of 8 weeks. The db/db mice display an increase in glomerular size and mesangial matrix by 5 to 6 months of age, and by 18 to 20 months, the glomerular and mesangial matrix enlargements become more remarkable, and thickening of the GBM is observed. In the oldest diabetic mice studied (up to 22 months of age), large subepithelial nodular densities were observed along with foot process fusion [90].

Other mouse models of type 2 diabetes more recently described are the eNOS$^{-/-}$/db/db mouse and the BTBR$^{ob/ob}$ mouse. The eNOS$^{-/-}$/db/db mouse is generated by backcrossing of eNOS knockout (KO) mouse on the C57/B6 background with db/db mouse on the C57BLKS/J background [91]. An association between decreased eNOS expression and development of advanced DN has been reported [91]. Thus, this double KO model exhibit obesity, hyperglycemia, hyperinsulinema, hypertension, dramatic albuminuria, and decreased GFR [92]. At kidney level, mice develop pathological changes of DN such as mesangiolysis, microaneurysms, increased mesangial matrix expansion, and nodular and diffuse glomerulosclerosis [91,92]. The BTBR$^{ob/ob}$ mouse model mimics progressive DN in BTBR strain with the ob/ob mutation [93]. It is characterized by insulin resistance, hyperinsulinemia, pancreatic islet hypertrophy, severe hyperglycemia, obesity, hypercholesterolemia, and elevated triglycerides. This mouse model rapidly develops pathological changes of both early and advanced human DN [94]. Glomerular hypertrophy, marked expansion of mesangial matrix, mesangiolysis, capillary basement membrane thickening, and loss of podocytes have been identified in this model [94].

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Table 10. Murine models of type 2 DN.

Model	Mechanism	Phenotypic alterations	Kidney pathology
db/db	G-to-T mutation in the leptin receptor gene, responsible for regulating sensation of satiety	Obesity Hyperglycemia Insulin resistance Polydipsia Polyuria Polyphagia Albuminuria	Glomerular hypertrophy Mesangial expansion GBM thickening
ob/ob	Spontaneous recessive mutation in leptin	Obesity Hyperglycemia Polyphagia Albuminuria	Mild renal structural changes
KK-Ay	Dominant mutation in agouti yellow (ay) gene, that alters normal control of body weight	Obesity Hyperglycemia Hypertriglyceridemia Albuminuria Obstructive uropathy	Glomerular hypertrophy Mesangial expansion Segmental proliferative glomerular nephritis
HFD	Obesity induced by high fat diet	Obesity Hyperglycemia Insulin resistance Hypertriglyceridemia Hypertension Albuminuria Metabolic syndrome	Mesangial expansion Increased glomerular tuft
eNOS$^{-/-}$/db/db	Backcrossing of eNOS KO with db/db	Obesity Hyperglycemia Polyuria Polydipsia Polyphagia Decline in GFR	Mesangiolysis Microaneurysms Mesangial expansion Nodular and diffuse glomerulosclerosis
BTBR$^{ob/ob}$	BTBR strain with ob/ob (leptin gene) mutation	Obesity Hyperglycemia Insulin resistance Albuminuria Hypercholesterolemia Polyuria Polydipsia Polyphagia	Mesangial expansion Mesangiolysis Glomerular hypertrophy GBM thickening Podocyte loss

Abbreviations: NZO, New Zealand Obese; HFD, high-fat diet; KO, knockout; GFR, glomerular filtration rate; GBM, glomerular basement membrane.

Current animal models of DN do not mimic advance renal failure resulting from diabetes, and associated increased risk for CV disease has been poorly characterized. In addition, the absence of renal failure may, in part, reflect an intrinsic resistance to nephropathy of the strains studied. Thus, new animal models to study DN are being developed and registered in the Diabetic Complications Consortium (https://www.diacomp.org).

D. Renin-angiotensin system. Angiotensin converting enzyme 2

D.I. Introduction

The RAS is a coordinated hormonal cascade involved in the homeostasis of peripheral vascular resistance as well as volume and electrolyte composition of body fluids. Thus, it exerts powerful influences to regulate many aspects of renal hemodynamic and transport function including the cortical and medullary circulations, glomerular hemodynamics, and the glomerular filtration coefficient in normal physiological and pathological conditions [95,96].

Classic RAS pathway is initiated by an aspartyl protease (renin) in the plasma that is released primarily from the juxtaglomerular cells on the afferent arterioles of the kidney [95,97]. The juxtaglomerular cells are thought to be derived from smooth muscle cells and contain numerous membrane-bound granules where renin is stored and synthesized [95]. Angiotensinogen (Agt) is primarily formed and then secreted by hepatic cells into the circulation. On release into the circulation, renin cleaves Agt at the N terminus to form the decapeptide, Ang I. Given that renin is released in response to various stimuli [98,99], large changes in plasma renin levels can occur rapidly, leading to changes in Ang I generation. Ang I is then converted to Ang II, due not only to the circulating dipeptidyl carboxypeptidase angiotensin-converting enzyme (ACE), but also to the widespread presence of ACE on endothelial cells of many vascular beds including the lung [100] (Figure 6).
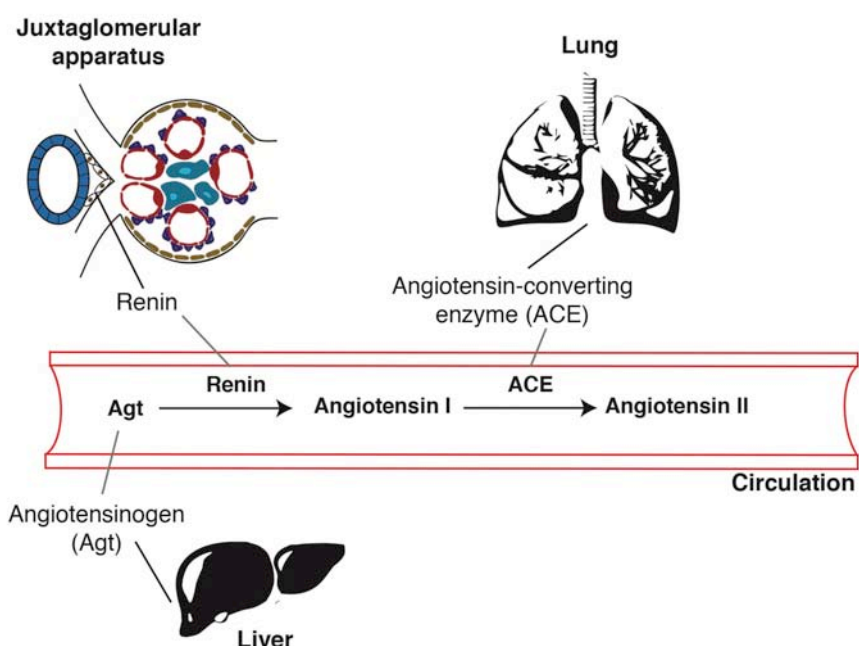
Figure 6. Schematic representation of classic RAS pathway. The RAS begins with the transformation of angiotensinogen (Agt) into angiotensin (Ang) I by renin secreted from juxtaglomerular apparatus cells. Ang I is then converted to Ang II by the angiotensin-converting enzyme (ACE).

In mammals, different isoforms of ACE have been described, expressed in somatic tissue and in germinal cells in the male testes [101]. The somatic form of ACE results in a two-domain protein with an N-terminal and C-terminal domain and with two M2-type zinc metallopeptidase motif, which are required for the peptidase catalytic activity [102] (Figure 7).

Figure 7. Domain structure of somatic ACE. The somatic form of ACE is a type 1 integral-membrane protein with an N-terminal ectodomain, a signal peptide (dark blue), two zinc-binding motifs (HEMGH), a transmembrane domain (red) and a short C-terminal cytoplasmatic domain (green).

The presence of local organ specific RAS has been demonstrated for the heart, large arteries and arterioles, kidneys, and other organs and their activation lead to structural and functional changes [103,104]. Although every organ system in the body has elements of the RAS, the kidney is unique in having every component of the RAS [103]. There is substantial evidence that the major fraction of Ang II present in renal tissues is generated from Agt locally produced by proximal tubular cells [105,106]. Renin secreted by the juxtaglomerular apparatus cells and delivered to the renal interstitium and vascular compartment also provides a pathway for the local generation of Ang I. ACE has been located in the proximal and distal tubules, the collecting ducts, and renal endothelial cells [107]. Therefore, all of the components

necessary to generate intrarenal Ang II are present along the nephron [108] (Figure 8).
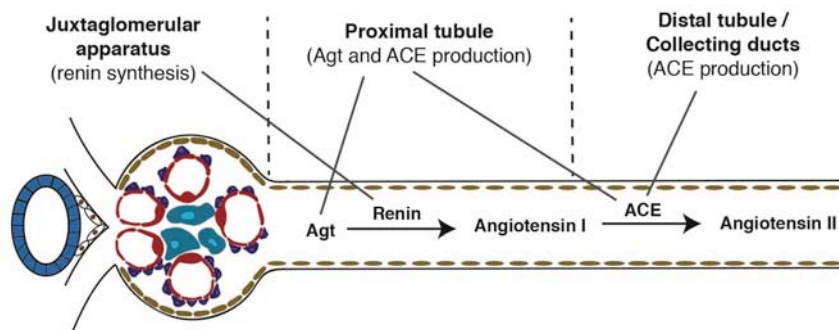
Figure 8. Schematic representation of intrarenal RAS. Renin secreted by the juxtaglomerular apparatus cells generates angiotensin (Ang) I from angiotensinogen (Agt) produced by proximal tubular cells. Then, Ang I is converted to Ang II by angiotensin-converting enzyme (ACE) located in the proximal and distal tubules.

Although there are other bioactive Ang peptides, Ang II is the most powerful biologically active product of the RAS. Ang II binds to different receptor subtypes such as angiotensin type 1 (AT_1) and angiotensin type 2 (AT_2) receptors. Through AT_1 receptor, Ang II mediates vasoconstriction, stimulates aldosterone production, enhances myocardial contractility, increases oxidative stress, and promotes inflammatory, atherogenic, and thrombotic states [103,109,110]. In addition to its physiological roles, locally produced Ang II induces inflammation, cell growth, apoptosis, migration and differentiation, regulates the gene expression of bioactive substances, and activates multiple intracellular signaling pathways, all of which might contribute to renal injury [103,111]. Ang II promotes podocyte injury indirectly by inducing cellular hypertrophy, increased apoptosis, and changes in the anionic charge of the glomerular basement membrane [111].

Besides the formation of Ang II by ACE, non-ACE dependent conversion of Ang I to Ang II have been described [112–114]. In normal human kidney, the study by Hollenberg et al. found that about 40% of Ang II generated locally could be attributed to the non-ACE pathway [114]. In kidney disease, the use of ACE inhibitors to blockade RAS and, thus reduce the formation of Ang II and its deleterious effects, has been shown to slow the progression of the disease but does not arrest disease progression to ESRD [115,116]. Therefore, these studies suggest a significance of an ACE-independent pathway for Ang II formation in disease progression.

D.II. Angiotensin converting enzyme (ACE) 2: localization and function

In 2000, a novel human cDNA homologous to ACE, ACE2, was identified among 19000 sequences obtained from a human cardiac left ventricle cDNA library that was prepared from the explant of a heart transplant recipient with idiopathic dilated cardiomyopathy [117]. Human ACE2 gene is localized in X chromosome (Xp22.2) and encodes for a 805 aminoacids protein that includes an N-terminal signal sequence, a metalloprotease zinc binding site (HEMGH), and a hydrophobic region near the C-terminus that is likely to serve as a membrane anchor (transmembrane domain). In comparison to ACE, ACE2 contains a single active-site domain and has 40% identity to the N-domain and C-domain of somatic ACE [117–119] (Figure 9).
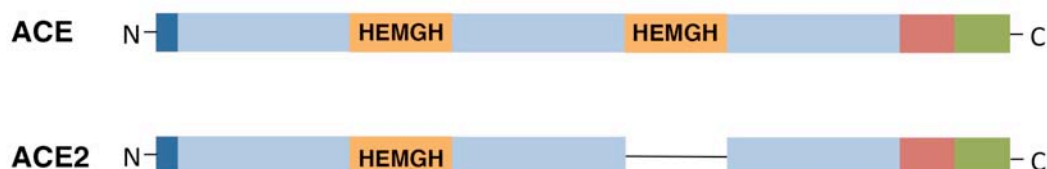
Figure 9. Domain structure of ACE and ACE2. Each protein is a type 1 integral-membrane protein with an N-terminal ectodomain, a short C-terminal cytoplasmatic domain (green), a signal peptide (dark blue), and a transmembrane domain (red). ACE contains two zinc-binding motifs (HEMGH), while ACE2 contains only one. HEMGH motifs form the active sites of the enzymes.

Initial gene expression studies by northern blot revealed that ACE2 was expressed only in heart, kidney, and testis [117]. Subsequent studies in mice and rats have demonstrated that ACE2 is also present in other organs such as lung, liver, brain, pancreas and colon [120–124]. ACE2 is involved in the generation of alternative Ang peptides in particular by conversion of Ang II to Ang 1-7 and Ang I to Ang 1-9. Thus, while ACE generates Ang II from Ang I through cleavage of the C-terminal dipeptide His-Leu, ACE2 catalyzes the conversion of Ang II into Ang 1-7 by removing the C-terminal amino acid phenylalanine. In addition, ACE2 can cleave the C-terminal residue of the decapeptide Ang I, thus generating the nonapeptide Ang 1-9, which may be subsequently converted to Ang 1-7 by ACE [99] (Figure 10).
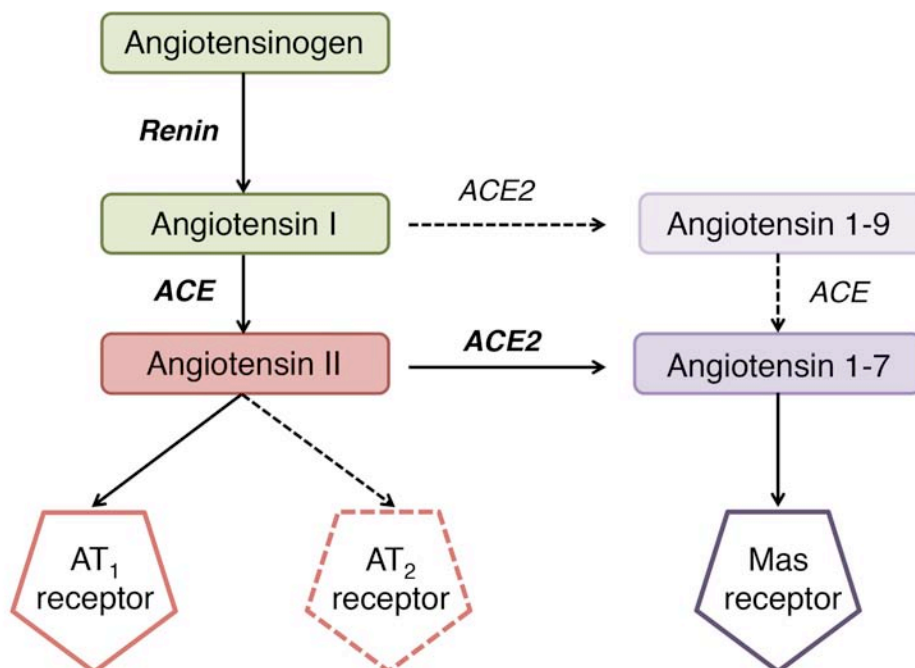
Figure 10. Schematic representation of RAS system. Solid arrows indicate substrates, enzymes and receptors of preferred action. Abbreviations: ACE, angiotensin-converting enzyme; AT_1 and AT_2 receptor, angiotensin II receptor 1 and 2.

Localization and function in the kidney

In rat kidney, mRNA for ACE2 has been detected in all nephron segments, except for the thick ascending limb of the Loop of Henle, with increased expression in the proximal tubule, the inner medullary collecting ducts an the vasa recta [125]. Within the glomerulus, ACE2 is mainly present in podocytes and mesangial cells. Studies in animal and human kidneys have demonstrated staining of ACE2 in both brush border and cytoplasm of proximal tubular cells [125–128].

Vickers et al. demonstrated that ACE2 hydrolyzes Ang II with high catalytic efficiency [129]. The biochemical evidence therefore indicates that ACE and ACE2 have complementary functions. ACE proteolysis generates Ang II, and ACE2 proteolysis degrades it to form Ang 1-7. In the kidney, Ang 1-7 counteracts the adverse effects of Ang II. Ang 1-7 acts via the G protein-coupled receptor Mas [130] to induce vasodilation and vascular protection, and anti-fibrotic, anti-proliferative and anti-inflammatory effects [131,132]. The role of ACE2 on shifting the balance of production away from Ang II toward Ang 1-7 was studied by Oudit et al., by investigating the impact of ACE2 deletion on kidney structure and function in mice. The kidneys of young ACE2 KO mice (3-month-old) showed no gross abnormalities and normal architecture of the cortex and the medulla. However, electron microscopy showed evidence of mesangial injury with small foci of fibrillar collagen deposition suggestive of an early disease process [133]. In adult male mice

(1-year-old) diffuse glomerulosclerosis, hyalinosis, and mesangial expansion were observed. In addition, immunehistochemical analysis showed increased staining of collagen I and III, fibronectin and α-smooth muscle actin. Clinically, albuminuria was detected in ACE2 KO mice [133]. Importantly, treatment with an ARB prevented glomerulosclerosis, hyalinosis, and mesangial expansion and there was complete resolution of the albuminuria, suggesting that the kidney injury is mediated largely through Ang II [133]. Subsequent experimental studies have shown that treatment with exogenous human recombinant ACE2 slows the progression of DN by reducing the increase in albumin excretion in diabetic mice. Glomerular hypertrophy and mesangial expansion were also reduced by human recombinant ACE2, confirming that modulation of angiotensin peptide metabolisms and its downstream effects can attenuate diabetic kidney injury [134].

Shedding of ACE2

Donoghue and Tipnis identified ACE2 as the first known human homologue of ACE and demonstrated the presence of a soluble form of ACE2 simultaneously [117,118]. In these studies, Chinese Hamster Ovary (CHO) cells were transiently transfected with expression plasmids containing ACE2 or no insert. While no protein expression was detected in media taken from untransfected CHO cells, an immunoreactive band was detected in the medium of ACE2-transfected cells [117,118]. In addition, the protein band obtained in the medium of ACE2-transfected cells was slightly smaller than the whole-cell lysate band, indicating that the full-length ACE2 is processed in CHO cells to generate a secreted form [117].

It is becoming increasingly apparent that the proteolytic shedding of cell surface proteins is an important mechanism regulating their expression and function. This ectodomain shedding event has been observed for a variety of membrane proteins with distinct functions, including cytokines (tumor necrosis factor-α (TNFα)), enzymes (ACE, ß-site amyloid cleaving enzyme (BACE)), and adhesion molecules (L-selectin) [135–137]. The first protease shown to be responsible for a particular shedding event was the so-called tumor necrosis factor-α converting enzyme (TACE) [135,138]. TACE, also known as ADAM17, belongs to the "a disintegrin and metalloprotease" family, modular type 1 transmembrane proteins that, in addition to the catalytic domain, contain a disintegrin and an EGF-like domain involved in protein-protein interactions (Figure 11).

Figure 11. Domain structure of TACE (ADAM17). ADAM17 is a type 1 integral-membrane protein with an N-terminal ectodomain, a signal peptide (dark blue), a prodomain (blue), a metalloprotease domain (yellow), a disintegrin domain (grey), an EGF-like domain (orange), a transmembrane domain (red) and a C-terminal cytoplasmatic domain (green).

A total of 33 metalloprotease disintegrins have been identified, but only 18 of them are predicted to function as proteases, while the rest do not contain the consensus Zn-binding domain, indicating that they are not active proteases. The shedding of TNFα is inhibited by metalloprotease inhibitors. Specifically, the endogenous metalloprotease inhibitor TIMP-3 inhibits ADAM17 activity [139]. Besides TNFα, ADAM17 is also capable of shedding soluble TNF receptors, L-selectin and TGFα [140].

The study by Lambert et al. was the first providing evidence that a catalytic active soluble form of ACE2 is cleaved from the membrane and that ADAM17 is involved in regulating its shedding [141]. *In vitro* studies in Human Embryonic Kidney 293 (HEK293) cells and in hepatocyte cellular carcinoma (Huh7) cells showed that ectodomain shedding of ACE2 was rapidly stimulated by the phorbol ester PMA, with a corresponding increase in ACE2 activity in the media that was decreased with the use of hydroxamic acid-based metalloproteinase inhibitors. Evidence of a role for ADAMs proteases in ACE2 shedding was provided by the results of experiments using synthetic inhibitors of ADAM10 and ADAM17. Interestingly, ACE2 shedding was strongly inhibited by the mixed ADAM10/ADAM17 inhibitor but was unaffected by the selective ADAM10 inhibitor (Figure 12A). Further evidences for the involvement of ADAM17 were provided by the results of experiments in which the cellular level of ADAM17 was depleted by specific RNA interference. Ablation of ADAM17 protein expression resulted in proportional reduction in stimulated ACE2 shedding (Figure 12B). These data implicated ADAM17 as a candidate *sheddase* for stimulating ACE2 shedding [141].
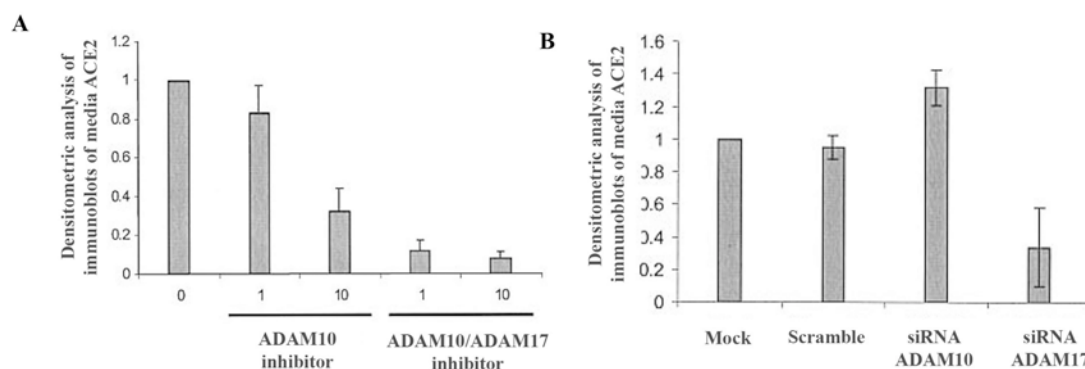
Figure 12. Densitometric analysis of immunoblots of ACE2 shedding. A) HEK cells were incubated in the presence of ADAM10 inhibitor or the mixed ADAM10/ADAM17 inhibitor. Densitometric analysis of immunoblots of ACE2 secreted in HEK cells media revealed that the mixed ADAM10/ADAM17 inhibitor effectively blocked ACE2 shedding stimulated by phorbol ester PMA. B) Ablation of ADAM17 expression by siRNA reduces stimulated ACE2 shedding. Densitometric analysis of immunoblots of ACE2 shedding showed a decrease in stimulated ACE2 shedding in HEK cells transfected with siRNA from ADAM17. Adapted from Lambert DW et al. 2005.

INTRODUCTION

A recent study by Chodavarapu et al. demonstrated that shedding of renal ACE2 into urine is increased in diabetic mice and that it correlated positively with the progression of diabetic renal injury represented by progressive albuminuria, mesangial matrix expansion and renal fibrosis [142]. In this study, immunostaining results demonstrated that ADAM17 colocalized with tubular ACE2 in diabetic kidney (Figure 13). Consequently, due to the actions of ADAM17, proteolytical active forms of ACE2 from the kidney are shed into urine of diabetic mice, suggesting that the loss of the renoprotective enzyme ACE2 could contribute to kidney damage [142].
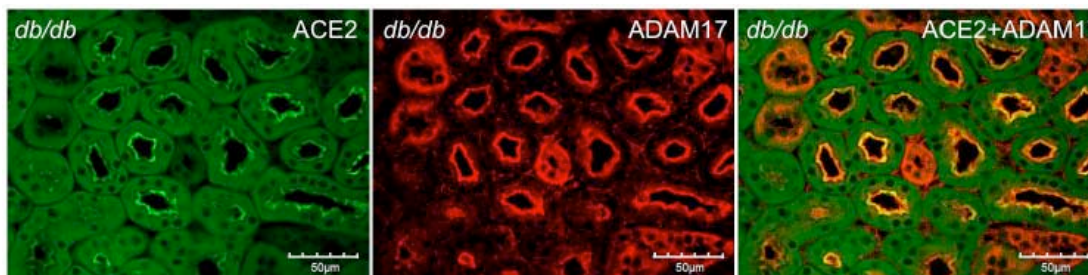
Figure 13. Immunofluorescence of ACE2 and ADAM17 in cortical tubules of db/db mice. From Chodavarapu H et al. 2013.

When blood glucose is controlled with anti-diabetic medication or daily exercise, there is a markedly attenuation in renal ADAM17 in db/db and Akita mice [142–144]. In addition, Xiao et al. were the first to demonstrate the shedding of active fragments of ACE2 from kidney proximal tubular cells and that high glucose (and to a lesser extent Ang II), stimulated ACE2 release from proximal tubular cells in an ADAM17 dependent-manner [145]. None of these works have described a pharmacological intervention against ADAM17. However, a recent study in the context of renal osteodystrophy has reported a direct effect of the synthetic vitamin D analog, paricalcitol, in inhibiting ADAM17 [146]. Furthermore, Morgado-Pascual et al. demonstrated inhibitory and protective effects of paricalcitol on ADAM17 in cultured tubular epithelial cells [147].

These studies suggest that hyperglycemia increases renal ADAM17 expression in coalition with a rise in urinary ACE2 excretion most likely due to increased shedding of renal ACE2 mediated by ADAM17 (Figure 14). Moreover, enhanced proximal tubular shedding of ACE2 fragments via ADAM17 could increase Ang II degrading capacity in the urine, and could serve as a biomarker of early kidney injury.
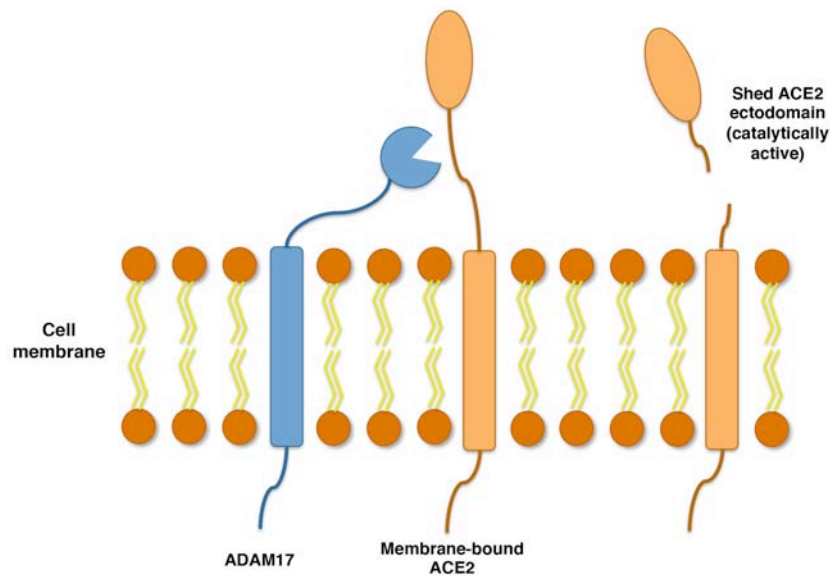
Figure 14. Schematic representation of ACE2 shedding by ADAM17. ACE2 undergoes shedding from the cell membrane by the action of the metalloprotease ADAM17 to release a catalytically active soluble form of ACE2.

D.III. ACE2 as a cardiovascular and renal marker: human and animal model evidences

ACE2 and cardiovascular disease

Several studies have demonstrated a relationship between ACE2 and CV disease. Burrell et al. revealed that ACE2 protein was present in viable myocardium, border zone, and the infarct, and was localized not only in endothelial cells and smooth muscle cells from intra-myocardial vessels, but also in cardiac myocytes [148]. Subsequent experimental studies have described the presence of ACE2 on the endothelial layer overlying neointima 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 [149]. In concordance, ACE2 has been also found to be expressed in human atherosclerotic plaques and located in different cell types present in the lesion, such as endothelial cells and macrophages [150]. The importance of ACE2 in cardiac function was strengthened by Crackower et al. who described cardiac dysfunction in ACE2 KO mice [119]. There was a 40% decrease in fractional shortening with slight ventricular dilatation. In addition, hearts from these ACE2 KO mice showed increased Ang II levels, suggesting that cardiac function is modulated by the balance between ACE and ACE2, and that the increase in local cardiac Ang II is involved in these abnormalities [119].

Accumulating evidence indicates that over-activity of cardiac RAS and myocardial Ang II production contributes to the progression of heart failure.

Thus, several studies have characterized ACE2 expression and activity in CV disease. In experimental myocardial infarction, increased cardiac ACE2 expression was found in the infarct zone and the surrounding ischemic zone [148]. Local up-regulation of ACE2 was also found in explanted human hearts with ischemic cardiomyopathy [148,151,152]. In experimental studies, a cumulative damage from Ang II, that caused an age-dependent cardiomyopathy, was observed in mice with deletion of ACE2 [153]. The potential for ACE2 to modulate cardiac function and remodeling is additionally suggested by the finding that lenti-viral vector encoding mouse ACE2 injected intracardially in Sprague-Dawley rats significantly attenuated cardiac hypertrophy and myocardial fibrosis induced by Ang II infusion [154]. These findings may imply that the up-regulation of ACE2 is a compensatory response to the ischemic insult and that the consequent increase in the vasodilator Ang 1-7 may confer cardio-protective effects.

Initial studies attempting to detect circulating ACE2 activity were able to detect it only in patients with CV disease [155]. The study by Lew et al. described the presence of an endogenous inhibitor of ACE2 that did not allow detection of the enzyme by catalytic activity assays. Removal of the inhibitor allowed ACE2 activity to be detected in human plasma samples from healthy volunteers [156]. Thus, subsequent studies demonstrated that circulating ACE2 activity was detected both in patients with CV disease and in healthy subjects and that it was increased in heart failure patients [156–158]. In concordance, circulating ACE2 activity was found to be up-regulated in the acute phase of ST-elevation myocardial infarction and it correlated with the infarct size [159]. In kidney transplant patients, Soler et al. demonstrated that circulating ACE2 activity was increased in those patients with previous history of ischemic heart disease [160].

ACE2 and kidney disease

ACE2 is highly expressed in the kidney, predominantly found in the proximal tubule brush border, in endothelial and smooth muscle cells of renal vessels, and in podocytes [120,126,128]. It has been reported that Ang II is increased in damaged tubules as a possible mediator of further renal damage in experimental and human renal disorders [161]. Thus, a disrupted balance between intrarenal ACE and ACE2 with consequent high levels of Ang II might therefore contribute to progressive renal damage. In experimental hypertension, renal ACE2 was decreased, and increased as a response to ACE inhibitors and ARBs [119,162,163]. In experimental diabetes there appears to be a different pattern on ACE2, depending on the mouse model and the kidney region [164]. Studies in ACE2 KO mice have shown an age-dependent glomerulosclerosis and albuminuria, probably caused by chronic exposure to increased circulating and tissue Ang II, as these abnormalities were abolished by ARB treatment [133]. In addition, Ye et al. supported the glomerulo-protective role of ACE2 by studies in chronic infusion of an ACE2 inhibitor. Mice treated with the ACE2 inhibitor showed increased albuminuria, resulting in increased deposition of glomerular fibronectin [128]. In

concordance with the study by Oudit et al., albuminuria could be prevented by an ARB [128]. Several studies have suggested that increased ACE2 activity tied with decreased ACE activity may reflect a protective mechanism by limiting the renal accumulation of Ang II and favoring Ang 1-7 formation [165,166].

Circulating ACE2 activity has been also detected in human and experimental kidney disease. Soro-Paavonen et al. demonstrated that circulating ACE2 activity was increased in male and female patients with diabetes, vascular complications and decreased estimated GFR, suggesting that counter-regulatory mechanisms are activated in kidney disease [167]. Sex differences in circulating ACE2 activity have been reported. Male patients show higher levels of circulating ACE2 activity in the context of DN, CKD, dialysis and transplantation [160,167,168].

D.IV. Modulation of ACE2 in diabetic nephropathy

One of the first studies analyzing the modulation of ACE2 in an STZ rat model showed that ACE2 protein levels were decreased by approximately 30% in the diabetic kidney [162]. In addition, ACE2 protein expression was decreased in renal tubules, while a significant number of glomerular cells were stained for this enzyme in the diabetic kidney. When a subgroup of STZ rats were treated with an ACE inhibitor (ramipril), there was a prevention of diabetes-associated decrease in renal ACE2 protein, although it did not appear to be mediated by an effect on ACE2 gene transcription, which remained unchanged with ramipril treatment [162]. Different results were obtained in studies with db/db mice [128,165]. The percentage of glomeruli with strong ACE2 staining was reduced, while ACE2 staining in tubules was increased in diabetic mice in comparison with controls. Interestingly, chronic ACE2 inhibition resulted in increased albuminuria and glomerular deposition of fibronectin in diabetic mice. The authors suggest that the effect of ACE2 inhibition is mediated via Ang II, because co-treatment with an ARB (telmisartan) prevented the increase in urinary albumin [128]. In a study analyzing the expression of ACE and ACE2 in diabetic mice, Ye et al. found that the relative abundance of ACE2 protein determined by Western blotting or by immunostaining was increased in kidney cortex from db/db mice at an early stage (8 weeks) [165]. The same group analyzed the enzymatic activity of ACE2 in STZ and db/db mice and described an increase in renal ACE2 activity that was associated with an increase of ACE2 protein but not ACE2 mRNA. This results are not consistent with the previous finding by Tikellis et al in 24 weeks diabetic rats [162], suggesting that differences may be due to the animal model used.

In a recent study in female NOD mice followed during 21 days (early stage) and 40 days (late stage), Riera et al. found that serum and urine ACE2 activity was increased in the NOD mouse model of diabetes at early and late stages of the disease. Interestingly, glycemic control by insulin administration

prevented the diabetes-induced increase in the serum and urine ACE2 activity. In kidney cortex, ACE2 activity and expression was also increased in both early and late stage [88]. Furthermore, it was demonstrated that serum and urine ACE2 in NOD mice correlated with increased UAE and increased GFR, as early markers of kidney involvement in diabetes [88]. Similar results were found in patients with type 1 diabetes, where serum ACE2 activity positively correlated with systolic blood pressure and diabetes duration among male [167]. Altogether, these results suggest that serum and urine ACE2 activity may be increased as a renoprotective mechanism and that this enzyme is already activated in an early stage of DN.

In human studies, Mizuiri et al. observed downregulated ACE2 expression and upregulated ACE expression in both the glomeruli and tubulointerstitium of diabetic patients with overt nephropathy, which led to the diabetic patients having significantly higher ACE/ACE2 ratios than the controls [169]. In this study, a positive correlation between the ACE/ACE2 ratio and the serum creatinine, fasting blood glucose, proteinuria and blood pressure values was determined. In addition, Reich et al. observed decreased ACE2 and increased ACE expression in the glomeruli and tubules in biopsy samples collected from patients with type 2 diabetes-induced kidney disease [170]. Conversely, Lely et al. detected upregulated ACE2 expression in glomeruli and tubule in all types of primary and secondary renal disease; however they only examined 8 diabetic patients [126].

D.V. Pharmacological modulation of renin-angiotensin system

The main goal for management for all patients with CKD includes reduction of the rate of progression of the disease to ESRD (delaying or preventing kidney failure) and prevention of CV events. Given that observational work has indicated an increased risk of progression of CKD and of kidney failure as blood pressure rises above 130/80 mmHg [171,172], effectively antihypertensive therapy is currently considered one of the most important treatment in CKD patients. Regarding DN, the basis for the prevention and control is the treatment of its known risk factors, namely hypertension, hyperglycemia and dyslipidemia. Thus, the goal treatment in DN is the prevention of the progression from micro to macroalbuminuria and also the occurrence of CV events. However, the above-mentioned strategies are not enough to delay DN progression and new therapeutic tools are needed.

Classical treatments

As it has been mentioned before, RAS is a hormonal cascade that regulates blood volume and arterial pressure to maintain adequate organ perfusion and is a major contributor involved in the progression of renal diseases. Indeed, continued activation of the RAS constricts renal arterioles and triggers pro-inflammatory and pro-fibrotic processes in the kidney contributing to progressive renal function loss [173]. Thus, blockade of RAS

has been largely proposed for the treatment in kidney diseases. Specifically, for more than 20 years, ACE inhibitors and ARBs have been considered the gold standard of treatment, given that their blood pressure-lowering effects have been demonstrated to improve clinical outcomes in both diabetic and non-diabetic renal disease [174–177]. Besides the blood pressure-lowering effects of these treatments, they have been also associated with lowering albuminuria. In the study by Hou et al. treatment of patients with advanced renal insufficiency without diabetes with the ACE inhibitor benazepril resulted in a 52% reduction in the level of proteinuria among treated patients as compared to a 20% in those assigned to placebo [175]. Likewise, benazepril was associated with a 24% reduction in the estimate decline in the GFR, with a median rate of decline of 6.8 mL/min/1.73m^2 per year as compared with 8.8 mL/min/1.73m^2 in the placebo group [175]. In a meta-analysis assessing RAS blockade therapy on reducing proteinuria in renal disease confirmed the antiproteinuric effect of ARBs across clinical subgroups, including patients with more or less proteinuria, and with and without diabetes [178]. In type 2 diabetic patients with DN a reduction of albuminuria during treatment with losartan has been demonstrated and it appeared to be beyond blood pressure reduction [179]. Post-hoc analysis confirmed that the risk for ESRD depended on albuminuria reduction, regardless of change in blood pressure [180]. In a randomized, double blind study aimed to assess the role of losartan in patients with type 2 diabetes and nephropathy, the risk of the primary end point, a composite of a doubling of the serum creatinine concentration, ESRD or death from any cause was reduced by 16% with losartan. This study also confirmed that the renal protection conferred by losartan was independent of blood pressure reduction [176]. Similarly, studies with irbesartan, showed a significant reduction in the rate of progression to clinical albuminuria and a slowing of the rate of progression of nephropathy, reflected in a significant increase in the time to a doubling of the serum creatinine concentration [177,181]. In both studies, these benefits appeared to be again independent of the systemic blood pressure [177,181]. A 5-year study evaluating the renoprotective effect of telmisartan and enalapril in hypertensive type 2 DN patients, showed that telmisartan was comparable to enalapril in reducing GFR decline [182].

Given that single blockade with ACE inhibitors or ARBs can achieve partial and not-durable suppression of the RAS system and that they interrupt the RAS at different levels, it has been hypothesized that dual blockage with ACE inhibitors and ARBs would be most beneficial in the management of progressive CKD [183]. One of the earliest studies in which dual blockade of RAS was assessed in patients with hypertension, microalbuminuria and non-insulin dependent diabetes, reported an additional 34% reduction in albuminuria versus ARB therapy and an additional 18% reduction versus ACE inhibitor therapy [184]. In the meta-analysis performed by Kunz et al. the concomitant therapy with ARB and ACE inhibitor led to greater reductions in proteinuria than monotherapy [178]. Recently, the ONTARGET trial included patients at risk for CV disease rather than kidney disease and randomly assigned to treatment with ramipril, telmisartan or the combination of both.

INTRODUCTION

Combination therapy resulted in a reduction in blood pressure and albuminuria, but it did not afford CV protection in this population [185]. Subsequent results of the ONTARGET trial showed that CV and kidney outcomes, such as dialysis or doubling of serum creatinine, did not differ between dual-therapy and monotherapy, but there was an increased risk of hypotensive symptoms, syncope and acute renal dysfunction with dual RAS blockade [186]. Additionally, it has been suggested that RAS blockade with ACE inhibitors and/or ARBs may not be effective probably due to incomplete blockade of the RAS, which leads to a compensatory rise in renin levels as a consequence of the disruption of the feedback inhibition of renin production [187,188]. Thus, high renin expression increases the risk of Ang II-dependent and independent organ damage.

Novel treatments

Given that RAS blockade with ACE inhibitors and/or ARBs might not be effective in some patients, there is a need for new therapeutic strategies to slow down the progression of ESRD [189]. In this regard, the direct renin inhibitor aliskiren was proposed as a first-line treatment for the progression of CKD and DN. Aliskiren inhibits the first and rate-limiting step in the RAS cascade, the conversion of angiotensinogen to Ang I, thereby reducing synthesis of all subsequent components of the cascade, except plasma renin concentration [190]. Initial studies showed that aliskiren decreased systolic and diastolic blood pressure (SBP and DBP) [191]. Additionally, co-treatment of aliskiren with furosemide in DN patients also reduced SBP and urinary ACR compared to baseline values [192]. Other studies confirmed the reduction in urinary ACR when patients were treated with aliskiren and ARB (losartan or olmesartan) [193,194]. Nakamura et al. reported that the combination therapy of aliskiren and olmesartan caused not only a reduction in proteinuria but also reductions of SBP and DBP in non-diabetic CKD patients [195]. In animal studies, Pilz et al. showed that double-transgenic rats (generation of large quantities of Ang II in the circulation, vasculature, heart and kidney) treated with aliskiren showed a decrease in SBP and albuminuria, and normalized serum creatinine [196]. Subsequent studies from the same group observed a reduction in albuminuria, TNFα, C-reactive protein and complement expression to control levels due to aliskiren treatment [197]. However results from the clinical trial ALTITUDE enrolling type 2 diabetic patients at high risk of CV events showed that the direct renin inhibitor aliskiren, when added to standard RAS blockade, did not reduce CV or renal outcomes and resulted in an increased number of adverse events. Consequently, the trial was stopped prematurely [198,199].

Vitamin D analogs have also been proposed to slow down the progression of CKD. The hormonal form of vitamin D, 1,25-dihydroxyvitamin D_3 (1,25(OH)$_2$D$_3$), is a negative endocrine regulator of the RAS [200,201]. Homozygous mutant mice lacking the vitamin D receptor (VDR) gene develop high renin hypertension and cardiac hypertrophy, suggesting that vitamin D supplement may be beneficial to the CV system [188,200,202,203]. It was

reported that treatment with vitamin D analogs, such as calcitriol, doxecalciferol or oxacalcitriol in subtotally nephrectomized rats resulted in reduction of proteinuria, mesangial cell proliferation, IL-6, glomerular inflammation and glomerulosclerosis [204].

Recent studies in the context of diabetes have shown that VDR-deficient diabetic mice develop a more severe nephropathy than wild-type mice [188,202]. The hallmarks of DN (proteinuria and glomerulosclerosis) were more severe in diabetic VDR-deficient mice and albuminuria was markedly more severe with an earlier onset in VDR KO mice compared to wild-type [188]. These results suggest a protective role against hyperglycemia-induced renal injury. As it has been mentioned before, suppression of the RAS after treatment with either ACE inhibitors or ARB remains incomplete, probably due to disruption of the short feedback loop by which Ang II normally inhibits the release of renin [187]. It has been demonstrated that combination therapy with an ARB and a vitamin D analog effectively blocks the development of DN in type 1 diabetes as a result of effective inhibition of renin and Ang II production within the kidney [188].

Paricalcitol is an activated vitamin D analog that stimulates the VDR at low concentrations and suppresses renin expression in mice without inducing hypercalcemia [205]. In experimental studies, administration of paricalcitol together with an ACE inhibitor resulted in reductions in the expression of proteins for extracellular matrix components, tubular epithelial-to-mesenchymal transition markers, and proinflammatory cytokines [206]. The non-calcemic actions of vitamin D analogs that may help protect the kidney, include regulation of the local RAS and the NF-kB pathway, which promote renal damage and the progression of kidney disease characterized by proteinuria, glomerulosclerosis and tubulointerstitial fibrosis [207]. In diabetic mice, the combination of losartan and paricalcitol prevented albuminuria, restored the glomerular filtration barrier structure, reduced glomerulosclerosis and suppressed pro-fibrotic and pro-inflammatory cytokines [188]. In the largest randomized trial of type 2 diabetes and albuminuria patients, the VITAL trial, a combination of paricalcitol and ACE inhibitors or ARBs was assessed. As a result, addition of paricalcitol to RAS inhibition safely lowered residual albuminuria in patients with DN [208].

II. HYPOTHESIS

II. HYPOTHESIS

DN is the leading cause of CKD in patients starting renal replacement therapy and is associated with increased CV mortality and an activation of circulating and intrarenal RAS.

Within the RAS, ACE2 plays a major role in kidney diseases by inducing the degradation of Ang II to Ang 1-7, which has vasodilator, anti-fibrotic, anti-proliferative and anti-inflammatory effects; thus counteracting the deleterious effects of Ang II. In addition, the metalloprotease ADAM17 is involved in regulating ACE2 shedding from the membrane. Circulating ACE2 has been associated with CV disease and renal progression in kidney disease, but its role in CKD patients without history of CV disease remains unknown.

Current therapies based on RAS blockade (ACE inhibitors, ARBs and direct renin inhibitors) are the first-line treatment for the progression of kidney disease. However, RAS blockade may not be effective in all patients, and there is a need for new therapeutic strategies to slow down the progression to ESRD. In this regard, the synthetic vitamin D analog, paricalcitol, in combination with the classical RAS blockade has shown prevention of albuminuria, reduction of glomerulosclerosis and suppression of pro-fibrotic and pro-inflammatory molecules, among others. However, the role of paricalcitol in modulating ACE2 in the NOD mouse model of type 1 DN has not been addressed.

Given the importance of RAS, and particularly of ACE2, in the progression of kidney disease, the hypotheses of this thesis are that (1) circulating ACE2 activity is altered in CKD patients without previous history of CV disease and it may correlate with classical CV risk factors and CV outcomes; and (2) paricalcitol treatment modulates circulating ACE2 activity associated to a modulation in the renal content of ADAM17.

III. AIMS

III. AIMS

The general aim of this thesis is to study the modulation of circulating ACE2 activity in renal disease. To accomplish this objective, two main studies have been performed, with the following specific aims:

1) HUMAN STUDY

- To analyze circulating ACE2 activity in CKD patients without previous history of CV disease included in the NEFRONA project.
- To study the association between circulating ACE2 activity and baseline clinical parameters.
- To study the association between circulating ACE2 activity and renal and CV progression during 2 and 4 years of follow-up.

2) EXPERIMENTAL STUDY

- To analyze enzymatic activity and expression of circulating, urinary and intrarenal ACE2 and its modulation by paricalcitol treatment in NOD mice.
- To study the influence of paricalcitol on other RAS elements.
- To study the renal content of ADAM17 and its modulation by paricalcitol.
- To study the effect of paricalcitol in modulating renal oxidative stress.
- To analyze the effect of paricalcitol in ACE2 expression in tubular cells.

IV. MATERIAL AND METHODS

IV. MATERIAL AND METHODS

A.Human study

A.I. Patients and study design

A total of 2572 subjects from the NEFRONA project were included in the study. The NEFRONA project is an observational, prospective (four-year follow-up) and multicenter study. From November 2009 to June 2011 patients with CKD (estimated GFR < 60 mL/min/1.73m^2) were recruited from outpatient nephrology clinic and dialysis centers across Spain. The selection of kidney patients was carried out using a consecutive sampling of the patients arriving from outpatient nephrology clinics, which represent the entire health care system of the Spanish public network. Patients without CKD were recruited from different Spanish community health centers and stratified with the same distribution by age and gender as the patients with CKD. The inclusion and exclusion criteria are detailed in Table 11 [209].

Table 11. Inclusion and exclusion criteria from the NEFRONA population.

Inclusion criteria	Exclusion criteria
· Male and female patients with CKD (GFR < 60 mL/min/1.73m^2), including patients in dialysis. · Age range: 18 – 75 years. · Absence of CV disease: angina pectoris, acute myocardial infarction, ischemic stroke, hemorrhagic stroke, abdominal aortic aneurysm and atherosclerosis	· Intercurrent disease that entails a lack of follow-up or a life expectancy of less than a year. · Patients with active infections and/or hospitalized in the last month. · Any type of transplantation or history of transplantation · Previous history of carotid artery disease · Human immunodeficiency virus infection · Pregnancy

Abbreviations: CKD, chronic kidney disease; GFR, glomerular filtration rate; CV, cardiovascular

The protocol was 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.

Patients from the NEFRONA Study were classified into three groups according to their GFR estimated by MDRD-4 (Table 12).

Table 12. Classification of patients.

	Control group (CONT)	Non-dialysis CKD stage 3 to 5 group (CKD3-5)	Dialysis (hemodialysis or peritoneal dialysis) group (CKD5D)
GFR	≥ 60 mL/min/1.73m^2	< 60 mL/min/1.73m^2	-
Number of patients	568	1458	546

Abbreviations: CKD, chronic kidney disease; GFR, glomerular filtration rate.

Recruiting investigators completed a questionnaire with the patients' clinical data, including socio-demographic variables, specific data of the kidney disease, family history of early CV disease, CV risk factors (namely smoking, diabetes, hypertension or dyslipidemia) and current medications. An itinerant team from the NEFRONA Study, comprising two technicians and a nurse, carried out a physical and a vascular examination that included height, weight and measurement of SBP and DBP, carotid and femoral ultrasonography and ankle brachial index (ABI) measurement. Biochemical parameters were obtained from a routine fasting blood test taken no more than three months apart from the vascular explorations. In hemodialysis patients, samples were obtained before the second dialysis session of the week. EDTA-anticoagulated plasma samples were collected from all patients and controls and sent to the biobank of the Renal Research Network (REDinRen), where they were centrifuged at 3000 g and stored at -80ºC [209]. Plasma samples were then sent from the REDinRen biobank to our laboratory for the determination of circulating ACE2 enzymatic activity.

To study the modulation of circulating ACE2 in the NEFRONA population, three analysis were performed at three time points: (a) baseline, (b) 2-year follow-up, and (c) 4-year follow-up.

a) Baseline analysis

At baseline the following variables were recorded and studied:
- Clinical variables: gender, age, body weight, history of diabetes, hypertension, dyslipidemia, and smoking (active smokers over the last month).
- Analytical variables: blood glucose, glycosylated hemoglobin, and lipid and anemia profiles.
- Current treatments: ACE inhibitors, ARBs, diabetes medication (insulin and oral antidiabetic drugs) and vitamin D analogues.
- Carotid and femoral ultrasonography: presence or absence of plaques (carotid and/or femoral).

MATERIAL AND METHODS

b) 2-year follow-up analysis

For the prospective analysis, a total of 1458 non-dialysis CKD3-5 patients were included. Variables assessed were: renal parameters (serum creatinine, GFR and need of renal replacement therapy), evaluation of silent atherosclerosis (carotid/femoral ultrasonography, atheromatous disease (AD), ABI, and intima-media thickness (IMT)), and CV events and mortality.

Renal parameters

Progression of kidney disease was evaluated according to doubling of serum creatinine or 50% decrease in estimated GFR calculated with the 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 GFR, patients that started dialysis or had a kidney transplant during the follow-up were excluded from the analysis (Figure 15).
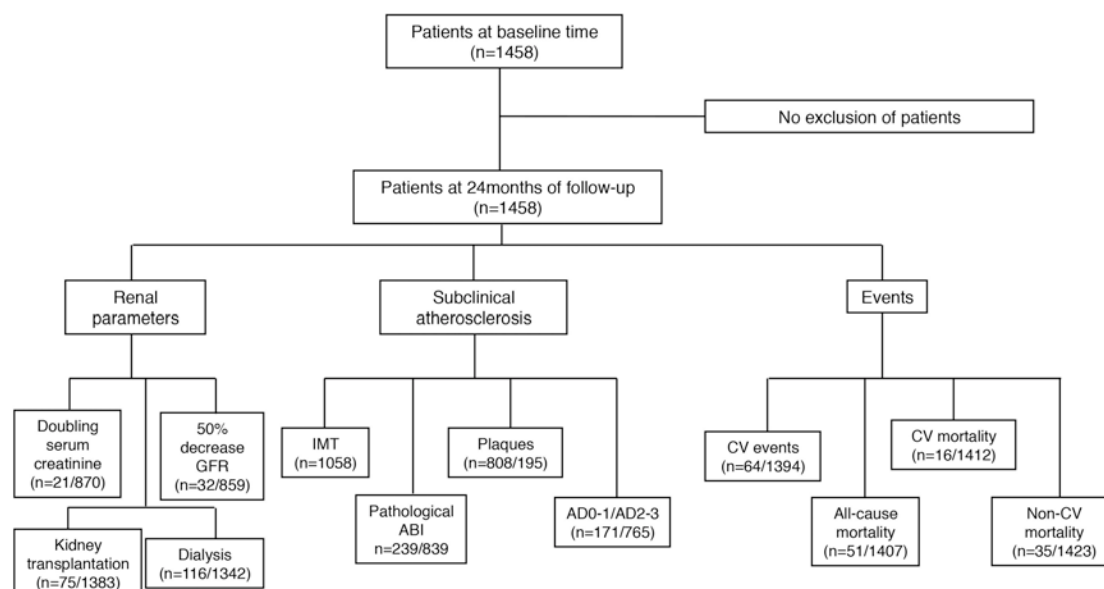
Figure 15. Schematic representation of patients included in the 2-year follow-up study analysis. Abbreviations: GFR, glomerular filtration rate; IMT, intima-media thickness; ABI, ankle-brachial index; AD, atheromatous disease; CV, cardiovascular.

Silent atherosclerosis

Subclinical atherosclerosis was evaluated as described by Junyent et al [209]. The itinerant team from the NEFRONA Study measured 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 plaques was performed using the semi-automatic software EchoPAC Dimension (General Electric Healthcare). The IMT was defined as the existing distance between the lumen-intima interface of the carotid artery and the media-adventitia interface of the distal wall. The plaques were defined as the IMT focal-

thickening with a height of >1.2mm or more than 50% of the adjacent IMT. To identify them in the entire accessible carotid/femoral area, type B longitudinal and transversal sections were carried out, followed by an examination with color Doppler sonography, adjusting the technical parameters (gain, repetition frequency of pulses and range of speeds) to the existing speeds in the blood vessel. The reading process was centralized and carried out by two independent observers from the NEFRONA Study who were unfamiliar with the participants' clinical characteristics. The quality of the reading and the intraobserver reliability was confirmed by the measurement of a sample of 20 individuals 3 to 5 times on different days (kappa coefficient = 1).

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 arteries, and left superficial femoral arteries. Patients were classified in three groups according to the number of territories with plaques: 0; 1-4; and ≥5. The increase in the number of territories with plaques from baseline to 24 months was also determined (Figure 15).

To assess the evolution of plaques from baseline time to 24 months, patients were classified according to the type of plaque and their evolution during the 24 months of follow-up (Figure 16). Regarding the type of plaques, they were classified in: any plaque (carotid and/or femoral), carotid plaque and femoral plaque. For the evolution of plaques, patients were classified in three groups: no plaque (patients without plaque at any time); *de novo* plaque (appearance of plaque during the 2-year of follow-up); and baseline plaque (patients with plaque at both baseline and 24 months).
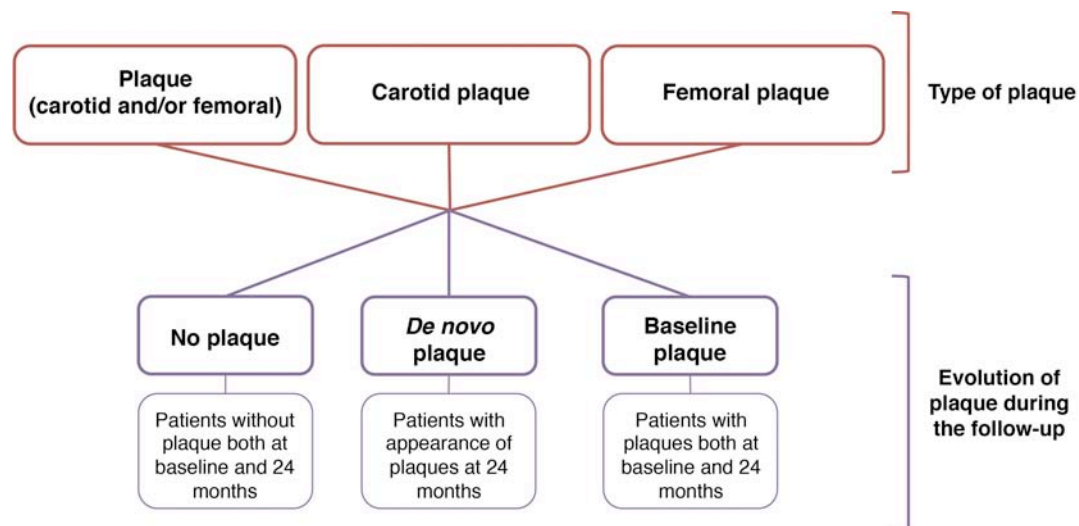
Figure 16. Classification of patients according to the type of plaques and their evolution during the follow-up period.

MATERIAL AND METHODS

Vascular Doppler MD2 Hungleight was used with an 8 MHz transducer and a cuff to manually measure blood pressure. The determination of blood pressure was performed in the brachial artery of both arms and in both feet. To calculate the ABI, the highest brachial blood pressure or the closest in time to the malleolar pressure in the ankle was used. A pathological ABI was defined as a value ≤ 0.9 (diagnostic of limb ischemia) or ≥ 1.4 (diagnostic of arterial incompressibility and stiffness, usually ascribed to vascular wall calcification) (Figure 15).

AD was scored into two groups according to the carotid ultrasonography findings and the ABI measurements: Stage 0-1 (AD 0-1) that included patients with ABI ≥ 0.7 and/or carotid IMT ≥ 90% according to reference interval (RI); and Stage 2-3 (AD 2-3) that included patients with ABI < 0.7 and/or carotid plaque with or without stenosis (Table 13) (Figure 15).

Table 13. Classification of atheromatous disease (AD) according to carotid ultrasonography and the ankle brachial index (ABI).

AD 0-1		AD 2-3	
AD 0	**AD 1**	**AD 2**	**AD 3**
ABI > 0.9 and IMT < 80% RI	ABI 0.7–0.9 and/or IMT ≥80% RI	Carotid plaque with stenosis <125cm/s	ABI < 0.7 and/or carotid plaque with stenosis ≥125cm/s

Abbreviations: IMT, intima-media thickness; RI, reference interval.

Events

All patients with CKD were monitored every six months for the appearance of CV disease, which was recorded according to the ninth version of the International Statistical Classification of Disease (ICD-9) [209]. Events were classified as CV events, CV mortality, non-CV mortality and all-cause mortality (CV and non-CV mortality). CV 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 CV mortality were also registered: myocardial ischemia and infarction, hyperkalemia or arrhythmia, cerebrovascular accident (ischemic or hemorrhagic), hemorrhage due to aneurysm rupture, mesenteric infarct and sudden death. Non-CV mortality causes included infections, tumors, accidents, kidney disease and non-determined or unknown (Figure 15).

c) 4-year follow-up analysis

For the 4-year follow-up analysis, non-dialysis CKD3-5 patients were included. At this time point, only CV events and mortality were registered. A total of 1237 patients were included in the analysis at 48 months of follow-up and 221 patients were not included due to kidney transplantation or lost of

follow-up (Figure 17). Variables assessed were: non-fatal and fatal CV event, non-CV mortality, all-cause mortality (CV and non-CV mortality), and all-cause mortality and non-fatal CV event.
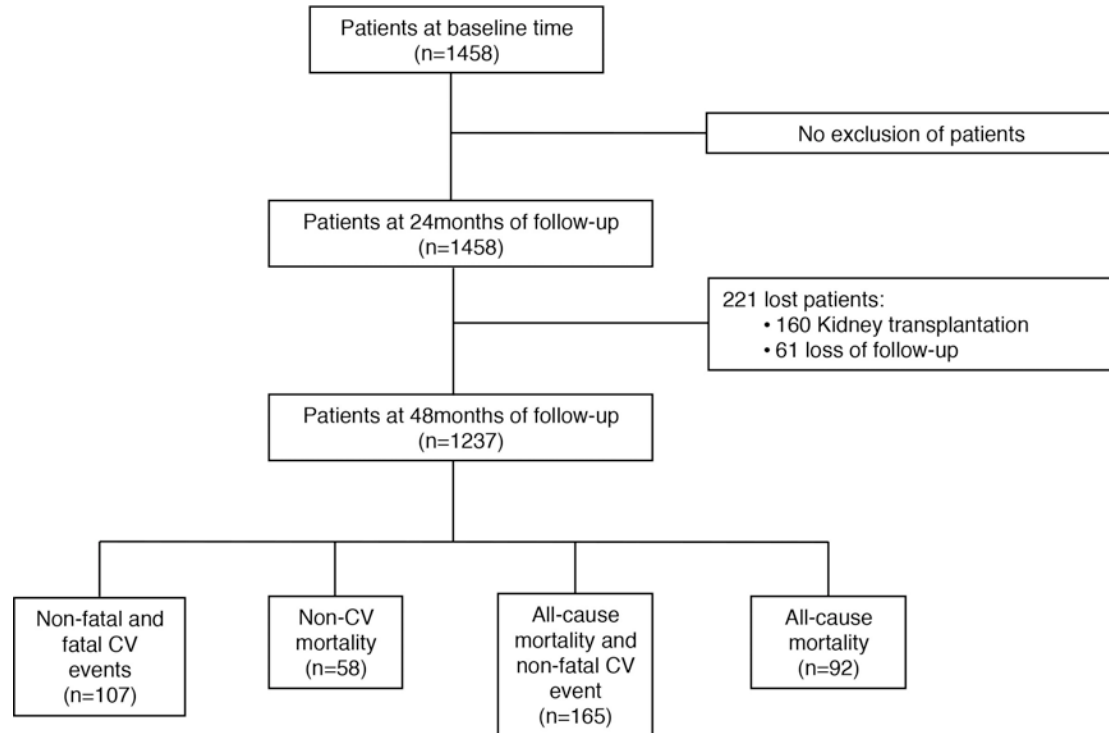
Figure 17. Schematic representation of patients included in the 4-year follow-up analysis. Abbreviations: CV, cardiovascular.

A.II. Circulating ACE2 enzymatic activity

The ACE2 fluorescent enzymatic assay protocol was performed using the ACE2-quenched fluorescent substrate Mca-Ala-Pro-Lys(Dnp)-OH (Enzo LifeSciences) and was measured at baseline time. The Dnp group quenches Mca fluorescence until the enzyme hydrolyzes the substrate by cleavage at Pro-Lys. Importantly, plasma samples were collected into tubes containing EDTA, which acts as a chelating agent of loosely bound metal ions such as Zn^{2+}. As it has been mentioned before (section D.II), ACE2 contains a metalloprotease zinc-binding site (HEMGH) that forms the active site of the enzyme. Therefore, EDTA present in the plasma samples binds to the zinc-binding site required for the enzymatic activity (Figure 18).
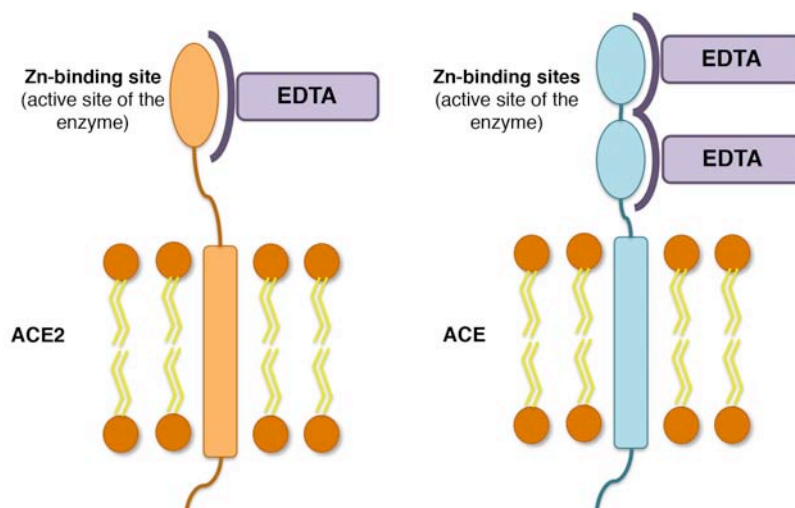
Figure 18. Inhibition of ACE2 and ACE enzymatic activities by binding of EDTA to the active site of the enzymes (Zn-binding site).

To avoid the binding of EDTA to the zinc ion required for the activity, calcium chloride ($CaCl_2$) and zinc chloride ($ZnCl_2$) were tested to revert the binding of EDTA to the catalytic site of ACE2. A total of 2 μL of plasma was incubated with appropriate buffer: 100 mM Tris-HCl pH 7.5, 600 mM NaCl, 10 μM $ZnCl_2$, in the presence of protease inhibitors: 100 μM captopril, 5μM amastatin, 5μM bestatin (Sigma-Aldrich) and 10 μM Z-Pro-prolinal (Enzo Life Sciences) in a 96-well plate. In addition, different concentrations of $ZnCl_2$ (0, 0.5, 1 and 3 mM) were added to the buffer to find the optimal concentration for the determination of ACE2 activity, which was found to be 0.5 mM of $ZnCl_2$. Samples were then incubated with 20 μM of the quenched fluorescent substrate in reaction buffer (final volume 100 μL) at 37 ºC for 16 hours. The plates were read using a fluorescence plate reader (Tecan Infinite 200, Germany) at Excitation 320 nm and Emission 400 nm. All experiments were carried out in duplicate, and results were expressed as RFU/μL plasma/hour.

A.III. Circulating ACE enzymatic activity

The ACE fluorescent enzymatic assay was performed using the synthetic substrate N-hippuryl-L-histidyl-L-leucine (HHL, Sigma-Aldrich). The enzyme present in the plasma samples cleaves the substrate at hippuryl-histidyl, to form the L-histidyl-L-leucine product that, in the presence of o-phthaldialdehyde (Sigma-Aldrich) forms a fluorescent adduct. A total of 0.83 μL of plasma were incubated with 73 μL of appropriate buffer (0.5 M borate buffer and 5.45 M HHL diluted in NaOH 0.025M) at 37 ºC for 25 minutes in a 96-well plate. Given that EDTA in the plasma samples binds to the zinc-binding site of ACE (Figure 18), different concentrations of $ZnCl_2$ (0, 7.81, 15.63 and 31.25 mM) and $CaCl_2$ (0.5, 1, 5 and 10 mM) were added to the buffer to find the optimal concentration for the determination of ACE activity. For the detection of ACE activity a concentration of 15.63mM of $ZnCl_2$ was

found to be optimal. The reaction was stopped by adding 180 µL of 0.28 M of NaOH. The fluorescent product was formed when 20 mg/mL of o-phthaldialdehyde in methanol was added and, after 10 minutes, the solution was acidified with 3 N HCl (Sigma-Aldrich). Fluorescence was read using Tecan Infinite 200 at Excitation 360 nm and Emission 485 nm. All experiments were carried out in duplicate, and results were expressed as RFU/µL plasma.

A.IV. Statistical analysis

Statistical analyses were performed using the SPSS version 18.0 for Windows and a $P<0.05$ was considered statistically significant. Normality of the continuous variables was assessed by normal probability plots and variables were expressed as mean ± standard error (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.

a) Baseline analysis: specific statistical methods

Paired case-control studies with an equal distribution of gender, diabetes, hypertension, dyslipidemia, smoking habits, weight (± 5 kg), and age (± 3 years) were performed using the R package version 3.0.2. A total of 3 paired-case control studies were carried out to compare:
- CONT versus CKD3-5 population: 280 pairs.
- CONT versus CKD5D population: 188 pairs.
- CKD3-5 versus CKD5D population: 360 pairs.

The intraclass correlation coefficient (ICC) was used to determine the concordance of the assay between the different human samples.

Finally, multiple linear regression analyses, using the natural logarithmic transformation of plasma ACE2 or ACE activities, were carried out to identify independent predictors of enzymatic activity.

b) 2-year follow-up analysis: specific statistical methods

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.

Receiver operating characteristic (ROC) curve analysis was generated from the logistic regression models with individual data available on 808 patients with presence of territories with plaques at 24 months of follow-up as the dependent variable. ROC curve was used to evaluate the prognostic ability and the optimal cut-off value of baseline circulating ACE2 activity as a biomarker of atherosclerosis at 2 years.

c) 4-year follow-up analysis: specific statistical methods

Survival was plotted on <u>Kaplan-Meier curves</u> and comparisons between groups were evaluated using log-rank tests. Probability of survival was stratified by the optimal cut-off value of baseline circulating ACE2 activity obtained in the ROC curve analysis at 2-years of follow-up. Overall survival was analyzed for the following events: non-fatal and fatal CV event, non-CV mortality, all-cause mortality, and all-cause mortality and non-fatal CV event.

A <u>multivariate Cox proportional hazards regression analysis</u> assessed time to event while controlling for risk factors, including age (<65 versus ≥65 years), gender, smoking history, diabetes, dyslipidemia, creatinine levels, GFR, baseline circulating ACE2 activity, body mass index (BMI), SBP, DBP, pulse pressure, glucose, total cholesterol, HDL and LDL cholesterol, glycosylated hemoglobin, hemoglobin, phosphorous, parathyroid hormone, 25-hydroxi vitamin D, 1,25-dihydroxi vitamin D and treatments (ACE inhibitors, ARBs and statins).

B. Experimental study

B.I. Animal model and experimental groups

Female NOD/ShiLtJ and control female NOR/LtJ mice (The Jackson Laboratory, Bar Harbor, ME) were used as a model of type 1 diabetes mellitus. As mentioned before, the development of insulin-dependent diabetes in NOD mice is preceded by insulinitis at 4-5 weeks of age with a marked sex difference in the incidence of diabetic symptoms in the NOD mouse: only 20% of males and 80% of females develop diabetes [85].

The mice were housed in groups of three per cage with *ad libitum* access to mouse chow and water under a 12:12 hour light:dark cycle. Animals were maintained under specific pathogen-free conditions in ventilated microisolators. The Institutional Animal care and Use Committee of Hospital del Mar-Institut Hospital del Mar d'Investigacions Mèdiques approved all procedures, and all experiments adhered to the Spanish Guide for the Care and Use of Laboratory Animals.

Experimental groups

Blood glucose levels were determined with ACCU-CHEKCompact (Roche, Germany) in NOD mice starting at 12 weeks of age. Diabetes was considered established after two consecutive values of > 13.87 mM. Diabetic mice were randomly assigned to one of five study groups and followed for 21 days (Figure 19):
- **CONT** (n=12): NOR mice that received vehicle (90:10 propylene glycol:ethanol).

- **C-PARI_H** (n=6): NOR mice that received 0.8 μg/kg (high dose) of paricalcitol.
- **NOD** (n=20): NOD mice that received vehicle.
- **NOD+PARI_L** (n=21): NOD mice that received 0.4 μg/kg (low dose) of paricalcitol.
- **NOD+PARI_H** (n=20): NOD mice that received 0.8 μg/kg of paricalcitol.
- **NOD+ALSK** (n=16): NOD mice that received the specific renin inhibitor aliskiren.
- **NOD+ALSK+PARI_L**: NOD mice that received 0.4 μg/kg of paricalcitol and aliskiren.
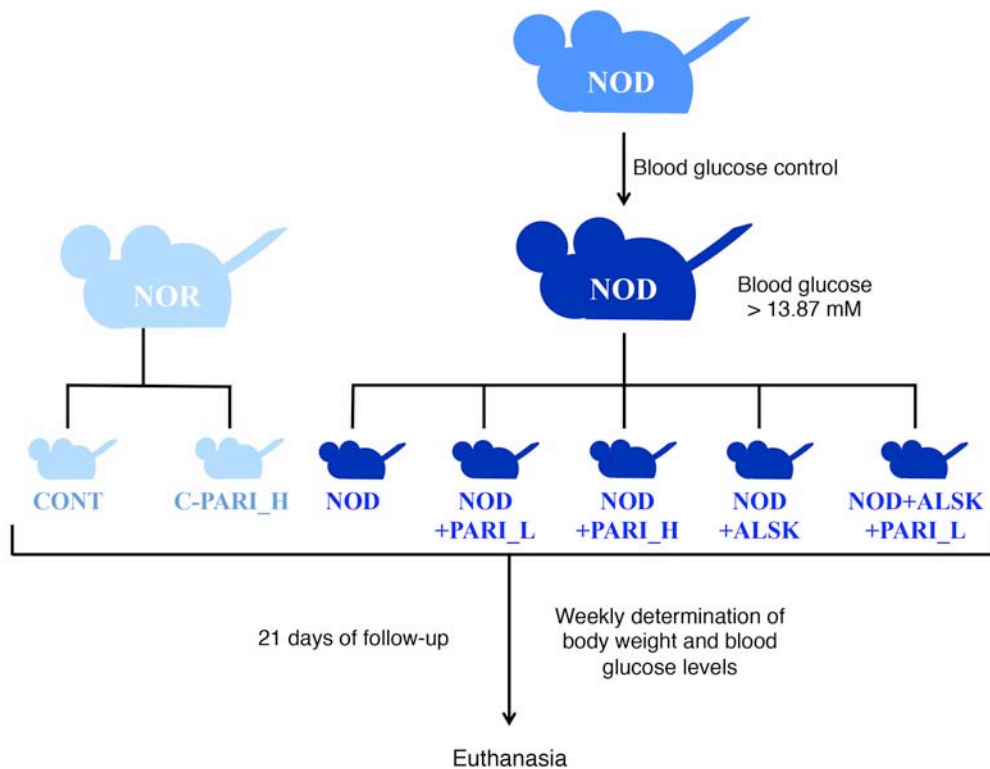
Figure 19. Experimental groups from the *in vivo* study and monitoring.

Paricalcitol was diluted in 90:10 propylene glycol:ethanol (Sigma-Aldrich) at a final concentration of 0.16 or 0.32 μg/mL depending on the treatment group. Animals received the assigned dose intraperitoneally thrice weekly. Aliskiren was diluted in saline at 189 mg/mL. Under sterile conditions in a vertical laminar flow hood, the aliskiren solution was delivered into a miniosmotic pump (ALZET; Durect Corp; USA) with a filling needle. Pumps were kept in eppendorf tubes with saline during 24 hours prior to implantation at 37ºC. This 24 hour incubation allowed the pumps to partially prime. Miniosmotic pumps were then implanted subcutaneously under ketamine/medetomidine anesthesia. After surgery, atipamezol was injected subcutaneously to reverse the effects of medetomidine. After 24 hours more

miniosmotic pumps started releasing Aliskiren at 0.11 µL/h. Thus, animals received 0.5 mg of aliskiren per day.

Mice monitoring

Mice were monitored for body weight and blood glucose levels weekly and then euthanized after 21 days of follow-up. Animals were anesthetized with sodium pentobarbital (200 mg/mL) diluted 1/10 in saline at a dose of 45 mg/kg. Blood was collected by intracardiac puncture. Afterward, mice were perfused with cold phosphate-saline buffer (PBS) by transcardiac puncture to flush out blood and kidneys and heart were removed, weighed, and processed for several purposes. Half of one kidney was fixed in 10% neutral-buffered formalin solution and processed for paraffin embedding according to standard procedures. The remaining tissue was snap-frozen and kept at -80ºC until it was used. Blood samples were centrifuged for 10 minutes at 8000 *g* at 10ºC and serum was transferred and maintained at -20ºC until used.

B.II. Blood pressure measurements

SBP, DBP and heart rate were measured using the noninvasive tail-cuff method (CODA™; Kent Scientific Corporation, Torrington, CT). Values were obtained from conscious-trained mice on six consecutive morning sessions by the same research assistant. Mice were placed on a heating platform at 33ºC in an acrylic restrainer, and a tail-cuff and pulse sensor was introduced along the tail. The tail cuff is connected to a cylinder of compressed air through an arrangement of inlet and outlet valves that allow inflation and deflation of the cuff at a constant rate. Results were calculated as the mean from the valid values of 30 measurements in each session and expressed in millimeter of mercury (mmHg), for SBP and DBP, and beats per minute (bpm), for heart rate.

B.III. Albuminuria determination

UAE was determined by the ACR on morning spot urine, collected during three consecutive days in microcentrifuge tubes and clarified by centrifugation. A commercial ELISA kit was used for albumin quantification (Albuwell M; Exocell, Philadelphia, PA) and a colorimetric assay kit was used to quantify creatinine levels (Creatinine Companion).

Albuwell M is an indirect competitive ELISA designed to monitor kidney function in the mouse by measurement of urinary albumin. The assay is performed in 96-well plates coated with mouse albumin (stationary phase), where sample (fluid phase) is added. The antigen in urine samples (albumin) is recognized by the primary antibody, a specific rabbit anti-murine albumin antibody. This primary antibody binds to the albumin immobilized in the

stationary phase or to that in the fluid phase. After washing, only the antibody-conjugate that bounds to the albumin of the stationary phase remains in the well. Primary antibody molecules are then labeled by the anti-rabbit IgG-HRP secondary antibody and detected using a chromogenic reaction. Given that it is an indirect competitive assay, albumin molecules in urine samples compete with the stationary albumin for binding to the primary antibody. Thus, color intensity is inversely proportional to the concentration in urine samples. In detail, 50 μL of standard curve point or diluted sample (1/13) were loaded in duplicate into albumin-coated wells. Afterwards, 50 μL of primary antibody was added to every well and incubated for 30 minutes at room temperature. Fluids were then removed from the wells and plate was washed 10 times with an appropriate wash buffer: 0.15 M NaCl and 0.05% Tween. 100 μL of secondary antibody (anti-rabbit HRP conjugate) were added to the wells and plate was covered and incubated for 30 minutes at room temperature. After the incubation, the plate was washed 10 times with wash buffer and 100 μL of TMB color developer was added. After 5 minutes of developing, addition of 100 μL of acid stopped the colorimetric reaction. Absorbance was then read in Tecan Infinite 200 at 450 nm.

Creatinine detection was performed in the same urine samples according to the classic technique described by Jaffe [210]. Picric acid in an alkaline medium reacts with creatinine from the urine sample to form a complex with the alkaline picrate. Intensity of the color formed is directly proportional to the amount of creatinine present in the sample. Briefly, 20 μL of standard curve point or diluted urine (1/20) were added to the plate and mixed with 100 μL of picrate solution. After 10 minutes of incubation at room temperature, absorbance was determined in Tecan Infinite 200 at 500 nm. Then, 100 μL of acid reagent were added to each of the wells, and read at 500 nm after 5 minutes of incubation at room temperature. Absorbance was determined by the following formula: Absorbance = Absorbance (alkaline picrate) – Absorbance (alkaline picrate+acid). ACR values were expressed as μg of albumin/mg of creatinine.

B.IV. ACE2 enzymatic activity

The ACE2 fluorescent enzymatic assay was performed as previously described using the ACE2-quenched fluorogenic substrate, Mca-Ala-Pro-Lys(Dnp)-OH (Enzo LifeSciences) (Section A.II from Materials and Methods). Enzymatic activity of ACE2 was detected in serum, urine and kidney tissue from all experimental groups.

For **serum** and **urine**, 2 μL of sample was incubated with assay buffer (100 mM Tris-HCl pH 7.5, 600 mM NaCl, 10 μM ZnCl$_2$) in the presence of protease inhibitors (100 μM captopril, 5μM amastatin, 5μM bestatin and 10 μM Z-Pro-prolinal) in a 96-well plate. Samples were incubated with 10 μM of the fluorogenic substrate in reaction buffer (final volume 100 μL) at 37 °C for

16 hours (serum sample) or 4 hours (urine sample). Plates were read in Tecan Infinite 200 at Excitation 320 nm and Emission 400 nm. Results were expressed as RFU/μL of sample/hour.

For **kidney** tissue, 50 mg of kidney cortex was homogenized in appropriate buffer: 50 mM HEPES pH 7.4, 150 mM NaCl, 0.5% Triton X-100, 0.025 mM ZnCl$_2$ (Sigma-Aldrich), 0.1 mM PefablocSC Plus and EDTA-free protease inhibitor cocktail tablet (Roche). Samples were centrifuged at 14000 g for 10 minutes at 4ºC. The protein concentration of each sample was determined using the MicroBCA assay kit (Thermo Scientific, Waltham, MA). This method uses bicinchoninic acid (BCA) as the detection reagent for Cu^{+1}, which is formed when Cu^{+2} is reduced by protein present in the sample in an alkaline environment. A purple-colored reaction product is formed by the chelation of two molecules of BCA with one Cu^{+1}. This complex exhibits a strong absorbance at 562 nm that is linear with increasing protein concentrations. Absorbance was read in Tecan Infinite 200 and results were expressed as μg/μL of protein.

After protein concentration measurement, samples were diluted in the same ACE2 assay buffer used for serum and urine samples. To each well, 0.25 μg of protein in 50 μL of assay buffer was added with or without the specific ACE2 inhibitor MLN-4760 (final concentration 10 μM) and the reaction was initiated by addition of 50 μL of the substrate at a final concentration of 5 μM. Activity was determined after 4 hours of incubation at 37ºC. Experiments were carried out in duplicate for each data point and results were expressed as RFU/μL of sample/hour, after subtraction of the inhibition value.

B.V. ACE enzymatic activity

The ACE fluorescent enzymatic assay was performed as previously described (Section A.III from Materials and Methods). Enzymatic activity of ACE was detected in serum and kidney tissue from all experimental groups.

For **serum**, 0.25 μL of sample was incubated with 75 μL of assay buffer (5 mM Hip-His-Leu in 0.4 M borate buffer and 0.9 M NaCl, pH 8.3) for 25 minutes at 37 ºC in a 96-well plate. The reaction was stopped by adding 180 μL of 0.28 M NaOH. The fluorescent product was formed when 20 mg/mL of o-phthaldialdehyde in methanol was added. After 10 minutes of incubation, the solution was acidified with 3 N HCl. Fluorescence was read using Tecan Infinite 200 at Excitation 360 nm and Emission 485 nm and results were expressed as RFU/μL of serum/minutes.

For **kidney** tissue, the homogenate obtained for ACE2 enzymatic activity detection was used. ACE activity was determined in tubes by adapting the protocol for serum samples. A total of 1 μg of renal protein was added to the reaction. After stopping the reaction with 3 N HCl, samples were clarified at

800 g for 5 minutes and dispensed into a 96-well plate to read fluorescence. Results were expressed as RFU/μg of protein.

B.VI. Serum renin activity

Serum renin activity was detected using a commercial kit (SensoLyte 520 Mouse ReninAssay Kit; AnaSpec, Belgium). This kit provides a continuous assay of mouse renin activity using a 5-FAM/QXL™ 520 fluorescence resonance energy transfer (FRET) peptide. In the FRET peptide, the fluorescence of 5-FAM is quenched by QXL™ 520. Upon cleavage into two separate fragments by mouse renin, the fluorescence of 5-FAM is recovered, and can be read at Excitation 490 nm and Emission 520 nm. A total of 5 μL of serum was incubated for 2 hours at 37ºC with the specific fluorogenic substrate provided with the kit. Fluorescence was detected in the Tecan Infinite 200 and results were expressed as RFU/5 μL.

B.VII. Quantification of Ang II and Ang 1-7

Cortical content of Ang II and Ang 1-7 were determined using commercial ELISA kits (Peninsula Laboratories). For this purpose, 50 mg of renal cortex was homogenized in 1 mL of pure methanol, centrifuged and evaporated at 3000 g at 37ºC for 1 hour. Samples were reconstituted with 320 μL of ELISA buffer (EIA buffer) and protein concentration was determined, as previously described, using the MicroBCA assay kit.

Same procedure was performed for quantification of renal Ang II and Ang 1-7. Briefly, 25 μL of antiserum (anti-Ang II or anti-Ang 1-7) was added to each well (except for the blank, where 25 μL of EIA buffer was added) and incubated for 1 hour at room temperature. Then, 50 μL of standard curve point, sample or EIA buffer (blank) was added and incubated for 2 hours at room temperature. After incubation, 25 μL of biotinylated tracer was added to each well and incubated overnight at 4ºC. The next day, the 96-well plate was incubated for 1 more hour at room temperature and then washed with EIA buffer (6 times). Once it was washed, 100 μL of secondary antibody (Streptavidin-HRP, diluted 1:200) was added and incubated for 1 hour at room temperature. Then, the 96-well plate was washed (6 times), 100 μL of TMB Solution was added to each well, incubated for 15 minutes at room temperature and the reaction was stopped with 100 μL HCl 2N. Absorbance was read in Tecan Infinite 200 at 450 nm. Results were expressed as picograms of peptide per milligram of total renal protein (pg/mg).

B.VIII. Renal cortex ADAM17 quantification

ADAM17 was determined in 5 μg of total protein extracts from renal cortex. Renal ADAM17 levels were determined using the Human TACE/ADAM17 DuoSet (R&D Systems, UK) following the manufacturer's instructions. In detail, 100 μL of diluted capture antibody was added to coat the 96-well plate and incubated at room temperature overnight. After incubation, wells were washed (3 times) with wash buffer and plate was then blocked by adding 300 μL of reagent diluent to each well and incubated for 1 hour at room temperature. Once the incubation was finished, plate was washed (3 times) and 100 μL of sample or standards in reagent diluent was added in each well and incubated for 2 hours at room temperature. The plate was washed and 100 μL of detection antibody was added and incubated for 2 hours. After repeating the washing procedure, 100 μL of Streptavidin-HRP conjugate was added, incubated for 20 minutes and washed. Finally, 100 μL of substrate solution was added to each well and incubated for 20 minutes. The reaction was stopped by adding 50 μL of stop solution and absorbance was read at 540 nm in Tecan Infinite 200. Results were expressed as picograms of peptide per milligram of total renal protein (pg/mg).

B.IX. Western blot analysis

Western blot analysis was performed to detect renal ACE2 and ACE protein expression. Western blot is an analytical technique that uses gel electrophoresis to separate denatured proteins by molecular weight. Then proteins are transferred to an absorbent membrane and incubated with specific antibodies.

For kidney tissue, the homogenate obtained for ACE2 enzymatic activity detection was used. A total of 20μg of protein was diluted in appropriate sample buffer (21mM TrisHCl a pH 6.8, 6% SDS, 34% glycerol, 19% β-mercaptoethanol and bromophenol blue) and denatured for 5 minutes at 100ºC. Samples were loaded in acrylamide/bisacrylamide (SDS-PAGE) gel (4% for stacking gel and 7% for separating gel) and electrophoresis was performed in the presence of a specific buffer (25mM Tris, 192mM Glycine and 0,1% SDS). After separation by molecular weight, samples were transferred into a PDVF membrane (GE Healthcare Life Science), previously activated with pure methanol. Transfer was performed in a semi-dry system (Trans-Blot® Turbo™, Biorad) during 30 minutes at 1 Ampere in appropriate buffer (25mM Tris, 192mM de Glycine pH 8.3 and 10% methanol). The membrane was incubated for 30 minutes in a blocking solution of 5% skimmed milk in TBS (Tris-buffered saline) and 0.1% Tween-20 (Sigma-Aldrich) (TBS-T0.1%). After blocking nonspecific binding sites of the membrane, antibody incubation was carried out under the conditions detailed in Table 14.

Table 14. Antibodies used for Western blot protein analysis.

Primary antibodies	Secondary antibodies
ACE2 1:2000 (ab15347, Abcam, UK)	**Rabbit IgG-HRP 1:3000** (Sigma-Aldrich)
ACE 1:1000 (F940, Bioworld Technology, St. Louis Park, MN)	**Rabbit IgG-HRP 1:3000** (Sigma-Aldrich)
β-actin 1:4000 (Sigma-Aldrich)	**Mouse IgG-HRP 1:15000** (Dako Agilent Technologies)

Protein bands were detected by chemiluminiscence (ECL Plus, GE Healthcare Life Science, Germany) on Curix RP2 film (Agfa Healthcare, Belgium). Band densitometry analyses were performed using ImageJ software (National Institutes of Health, Bethesda, MD) and results were expressed as ACE2 or ACE to β-actin ratio.

B.X. Immunohistochemistry for ACE2 and renin

Paraffin blocks were cut into 3-μm sections using microtome (Leica Biosystem). Sections were stretched in a water bath at 40ºC and collected into Superfrost microscope slides (Fischer Scientific). Samples were stored at 4ºC until use. For immunohistochemistry, excess of paraffin was melted by incubating the sections for 30 minutes in a stove at 60ºC. Sections were then deparaffinized in xylene (2x15 minutes) and rehydrated through graded alcohols at 1005 (2x10 minutes), 96% (1 minute), 70% (1 minute) and 50% (1 minute). Sections were then kept in water for 5 minutes.

ACE2 immunohistochemistry staining

Kidney sections were boiled in 10 mM sodium citrate solution (pH 6.0) for antigen retrieval. Unspecific binding blocking was performed in 1% bovine serum albumin (BSA, AppliChem) and 3% goat serum (Sigma-Aldrich) in PBS1X for 1 hour at room temperature. After washings, endogenous peroxidases were blocked in a 3% hydrogen peroxide (H_2O_2) solution, diluted in PBS1X, for 10 minutes in dark conditions. Primary antibody against ACE2 (MAB3437, R&D Systems) diluted 1:250 in blocking solution was incubated for 1 hour at room temperature. Secondary antibody (anti-rat IgG-HRP, Sigma-Aldrich) diluted 1:250 was incubated for 1 hour at room temperature. Proteins were visualized with the Dako REAL™ EnVision™/HRP Detection System in a 1:1 proportion (Dako REAL™ Substrate Buffer and Dako REAL™ DAB+ Chromogen). Sections were counterstained with hematoxylin, dehydrated and mounted with DPX mounting media.

MATERIAL AND METHODS

Renin immunohistochemistry staining

For renin staining sections were boiled in 10 mM sodium citrate solution (pH 6.0) for antigen retrieval. Endogenous peroxidases were then blocked in a 6% H_2O_2 solution, diluted in PBS1X, for 15 minutes in dark conditions. Unspecific binding blocking was performed in 3% BSA and 1% of goat serum in PBS1X for 30 minutes at room temperature. After 3 washing with PBS1X, endogenous immunoglobulins were blocked with fragment antigen-binding (Fab) fragment (AffiniPure Fab Fragment Goat Anti-Mouse IgG, Jackson ImmunoResearch, West Grove, PA) diluted 1/10 in PBS1X for 1 hour at room temperature. Primary antibody against Renin (Ab15347, Abcam) diluted 1:1000 in blocking solution was incubated for 1 hour at room temperature. Secondary antibody (Dako REAL™ EnVision™/HRP, Rabbit/Mouse (ENV), Dako, Denmark) was incubated for 45 minutes at room temperature. Proteins were visualized with the Dako REAL™ EnVision™/HRP Detection System in a 1:1 proportion (Dako REAL™ Substrate Buffer and Dako REAL™ DAB+ Chromogen). Sections were counterstained with hematoxylin, dehydrated and mounted with DPX mounting media.

The intensity of the staining, both for ACE2 and renin, was quantified with ImageJ software in a blinded fashion.

B.XI. Gene expression

Gene expression analyses were performed for ACE2, ACE and renin. RNA extraction, first-strand cDNA synthesis and real-time PCR of samples were carried out.

RNA extraction

Total RNA was extracted from 50 mg of renal cortex using Tripure Isolation Reagent (Roche). Tissue was homogenized with 800 μL of TriPure Isolation Reagent and incubated for 5 minutes at room temperature to ensure the complete dissociation of nucleoprotein complexes. Then 200 μL of chloroform was added to each tube, samples were shacked vigorously and incubated for 10 minutes at room temperature. To separate the solution in three phases (aqueous phase, RNA; interphase, DNA; and lower red organic phase, protein), samples were centrifuged at 12000 g for 15 minutes at 8ºC. After centrifugation, aqueous phase was transferred to a new centrifuge tube and 400 μL of isopropanol was added and incubated for 10 minutes at room temperature for precipitation of RNA. Samples were then centrifuged at 12000 g for 10 minutes at 8ºC, supernatant was discarded and 1 mL of ethanol 75% was added to the pellet and centrifuged at 7500 g for 5 minutes at 8ºC. This process was repeated twice and excess of ethanol from the RNA pellet was removed by air-drying for 30 minutes. Finally, RNA pellet was resuspended in RNase-free water.

RNA quantification and retrotranscription

RNA quantity and purity were analyzed with NanoDrop (ND-1000V3.3). First-strand cDNAs were synthesized from 1 μg of RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Briefly, appropriate volume of RNA was mixed with 10 μL of RT Master Mix 2x to obtain a final volume reaction of 20 μL (Table 15).

Table 15. Components of RT Master Mix 2x.

	RT Master Mix 2x
10X RT Buffer	2.0 μL
25X dNTP Mix (100mM)	0.8 μL
10X RT Random Primers	2.0 μL
MultiScribe™ Reverse Transcriptase	1.0 μL
RNase Inhibitor	1.0 μL
Nuclease-free H$_2$O	3.2 μL

Retrotranscription was performed in a thermocycler (TProfessional Basic, Biometra) by incubating for 10 minutes at 25ºC, 120 minutes at 37ºC and 5 minutes at 85ºC.

Real-time PCR

The polymerase chain reaction (PCR) is a technique for amplifying DNA that generates thousands to millions of copies of a particular DNA sequence. To perform PCR, primers containing sequences complementary to the target region, deoxynucleoside triphosphates (dNTPs) from which a new DNA strand is synthesized, and Taq polymerase are necessary. The method relies on thermal cycling, consisting of cycles of repeated heating and cooling of the reaction for DNA melting, primers annealing and enzymatic elongation of the newly synthetized DNA strain. As PCR progresses, the DNA generated is itself used as a template for replication, setting in motion a chain reaction in which the DNA template is exponentially amplified. In the real-time PCR (qPCR) the same methodology is used but a fluorophore is added to this mixture. In the thermal cycler that contains sensors for measuring the fluorescence of the fluorophore, the rate of generation of the amplified product at each PCR cycle can be obtained. Real-time PCR was performed in the Light cycler 489 System (Roche) following specific settings (Table 16) and using SYBR Green I Master, which binds all double-stranded DNA that are being generated throughout the cycles.

Table 16. Real-time PCR settings in the Light cycler 489 System. After the amplification cycles are completed, melting step is performed in order to assess that the PCR has produced single, specific products.

Step	Temperature	Ramp Rate	Time
Pre-incubation	95ºC	4.8ºC/s	5 minutes
Amplification (45 cycles)			
a)	95ºC	4.8ºC/S	10 seconds
b)	58ºC	2.5ºC/s	20 seconds
c)	72ºC	4.8ºC/s	20 seconds
Melting			
a)	95ºC	4.8ºC/s	5 seconds
b)	58ºC	2.5ºC/s	1 minute
c)	95ºC	0.11ºC/s	-
Cooling	40ºC	2.5ºC/s	10 seconds

The sequences of primers were as follows (Table 17). Relative quantification of gene expression was performed to quantify differences in the expression level of Ace2, Ace and Renin between different samples. Quantification of target genes were normalized by an endogenous control or housekeeping gene (β-actin).

Table 17. Primers used for ACE2, ACE, Renin and β-actin gene expression.

Gene	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
Ace2	ACCCTTCCTACATCAGCCCCACTG	TGTCCAAAATCTACCCCACATAT
Ace	CGCCGCTATGGGGACAAATA	ATGTCTCCCAGCAAATGGGC
Renin	TACGAGTCCCGGAATTCAAC	AGGAAGCCCAGGATGTTCTT
β-actin	TCACCATCCACTACGGATCA	CACAGTGATTCCACCCACAG

B.XII. Oxidative stress

Indirect markers, such as peroxynitrite production, generally identify oxidative stress. For this reason, nitrotyrosine staining, used to evaluate superoxide and peroxynitrite levels in renal tissues, and serum H_2O_2 content, as a byproduct of the superoxide dismutase oxidative stress response, were determined (Figure 20).
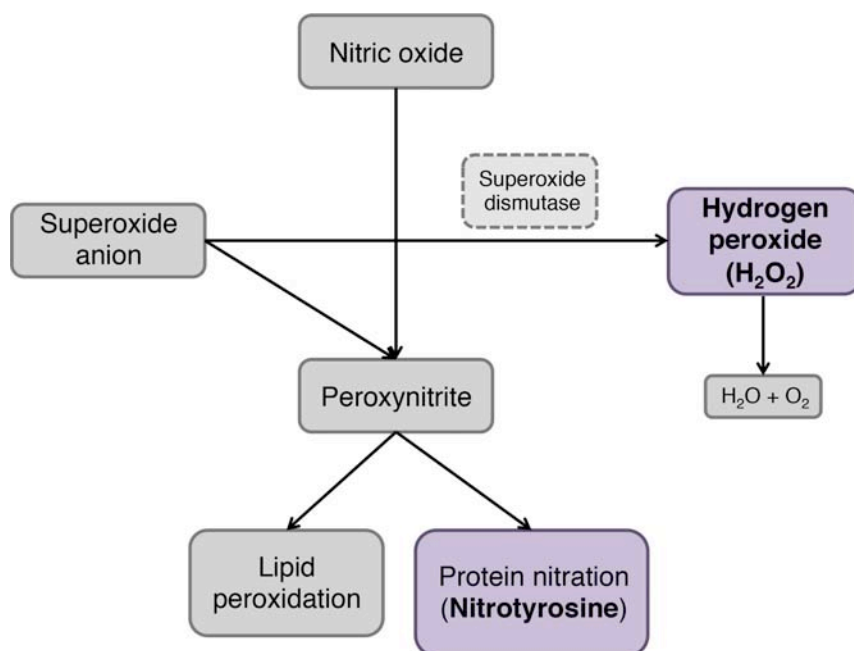
Figure 20. Schematic and simplified representation of oxidative stress pathway. Reactive oxygen species (ROS) include superoxide anion, hydrogen peroxide and peroxynitrite. Peroxynitrite is a potent oxidizing agent, generated from nitric oxide, which can result in protein and lipid damage by protein nitration and lipid peroxidation. Thus, nitrotyrosine is considered as a biomarker of oxidative stress. Hydrogen peroxide is formed by the enzyme superoxide dismutase, which catalyzes the dismutation of the superoxide anion into hydrogen peroxide. Hydrogen peroxide is thermodynamically unstable and decomposes to form water and oxygen, thus inducing oxidative stress.

Nitrotyrosine immunohistochemistry staining

Paraffin-embedded tissues were cut into 3-μm sections using microtome. Sections were then deparaffinized, rehydrated as mentioned before, and boiled in 10 mM sodium citrate solution (pH 6.0) for antigen retrieval. Endogenous peroxidases were blocked in a 3% H_2O_2 solution, diluted in PBS1X for 20 minutes in dark conditions. After 3 washing with PBS1X, unspecific binding blocking was performed in 3% bovine serum albumin (BSA, AppliChem) and 3% of goat serum (Sigma-Aldrich) in PBS1X for 30 minutes at room temperature. Primary antibody against nitrotyrosine (06-284, Merck Millipore, Germany) diluted 1:500 in blocking solution, was incubated for 1 hour at room temperature. Secondary antibody (Dako EnVision+ System-HRP Labelled Polymer Anti-Rabbit, Dako, Denmark) was incubated for 45 minutes at room temperature. Proteins were visualized with the Dako REAL™ EnVision™/HRP Detection System in a 1:1 proportion (Dako REAL™ Substrate Buffer and Dako REAL™ DAB+ Chromogen). Sections were counterstained with hematoxylin, dehydrated and mounted with DPX mounting media.

Ten microphotographs at x40 were taken for each sample, and brown-stained areas were quantified with Image J software. Data were expressed as arbitrary units of mean gray value (AU of MGV).

Serum hydrogen peroxide content

H_2O_2 content in serum samples was detected using the Amplex Red Assay kit (Invitrogen, UK). A total of 2.5 μL of serum (diluted in 47.5 μL of reaction buffer 1X) and 50 μL of each standard curve point were added to the 96-well plate. The reaction began with the addition of the working solution (100 μM Amplex Red Reagent and 0.2U/mL HRP) and was incubated during 30 minutes at room temperature in dark conditions. Finally, absorbance was read in Tecan Infinite 200 at 560 nm. Experiments were carried out in duplicate and results were expressed in micromole (μM).

B.XIII. Cell culture studies

Cell culture studies were performed using a cortical proximal tubular epithelial cell line (MTC, kind gift from Dr. López Novoa). Cells were grown in complete medium (RPMI 1640 (Invitrogen) with 10% inactivated fetal bovine serum (FBS), 1% penicillin/streptomycin and 1% glutamine) in 5% CO_2 at 37ºC. When cells were confluent, they were trypsinized with 1 mL trypsin to dissociate adherent cells. Trypsin was deactivated with 10 mL of complete medium and cells were centrifuged at 1000 rpm for 5 minutes. Supernatant was discarded and the pellet of cells was resuspended in 4 mL of complete medium. After resuspension, cells were counted in a Neubauer chamber and seeded in 24-well culture plates (Corning® Costar® Cell Culture Plates, Life Sciences). In each well, a total of 4000 cells were seeded for 24 hours on sterilized glass coverslips. Cells were serum-starved for 24 hours and incubated with 0 (CONT), 0.04 (Pari_0.04), 0.4 (Pari_0.4) and 4 (Pari_4) ng/mL of paricalcitol for 24 hours more (Figure 21). Media in the experiments contained high concentrations of D(+)-glucose (25mM) (Sigma-Aldrich). After incubation with paricalcitol, cells in sterilized glass coverslips were washed and kept at -20ºC in pure methanol, until the immunofluorescence of ACE2 was performed.
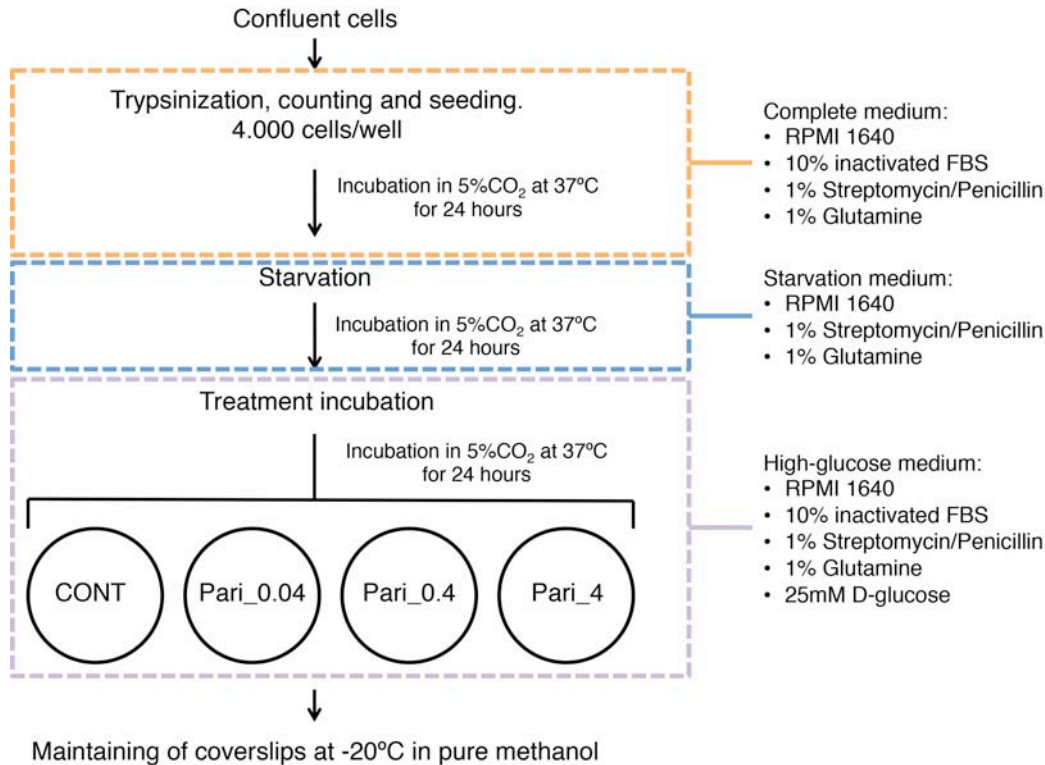
Figure 21. Schematic representation of cell culture study.

Immunofluorescence of ACE2

Coverslips for immunofluorescence of ACE2 were maintained in pure methanol at -20ºC until use. For immunofluorescence staining, coverslips were washed with cold PBS and incubated for 45 minutes at room temperature with the blocking solution (3% BSA in PBS with 0.1% Tween-20). Then, incubation with 200 μL of ACE2 primary antibody (MAB3437, R&D Systems) diluted 1:500 was performed. After incubation of primary antibody and 3 washings with PBS, 200 μL of AlexaFluor 555 secondary antibody (Life Technologies) diluted 1:2000 was added to each coverslip. Finally, coverslips were mounted on glass slides using an appropriate solution consisting in 90% Mowiol, 10% DABCO and 0.2 μL DAPI (all from Sigma-Aldrich). Cell staining was examined with a high-resolution spectral confocal Leica TCS SPE microscope. Intensity of staining was measured with Image J software and results were expressed as arbitrary units of fluorescence per cell. All analyses were performed in a blinded fashion.

B.XIV. Statistical analysis

Statistical analyses were performed using the SPSS version 18.0. Because the sample size was small, non-parametric tests were conducted. A Mann-Whitney test was used to compare between two groups. In addition, a Kruskal-Wallis test was performed for multiple comparisons in the study. Significance was defined as $P < 0.05$ and values were expressed as mean ± SE.

V. RESULTS

V. RESULTS

A.Human study

A.I. Patients characteristics

A total of 2572 patients from the NEFRONA Study were included in the analysis. Of them, 568 were CONT subjects and 2004 CKD patients without previous history of CV disease, divided into those not requiring dialysis (CKD3-5, n=1458) and those in dialysis, either hemodialysis or peritoneal dialysis (CKD5D, n=546). Characteristics of study subjects are shown in Table 18. In general, CONT patients had less prevalence of diabetes, hypertension, dyslipidemia, and smoking habits than CKD patients (both CKD3-5 and CKD5D). As expected, the percentage of patients in RAS blockade therapy was also lower in CONT than in CKD subjects. Interestingly, CKD5D patients were younger, thinner and had less prevalence of diabetes, hypertension and dyslipidemia than CKD3-5 patients.

Table 18. Clinical characteristics of study cohorts.

|	Total population (n=2572)	CONT (n=568)	CKD3-5 (n=1458)	CKD5D (n=546)	P-value		
					CONT versus CKD3-5	CONT versus CKD5D	CKD3-5 versus CKD5D
Age (years)	56.8±12.6	54.3±11.5	59.5±11.9	52.86±13.8	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±15.2	77.2±15.0	78.0±14.8	72.40±15.7	p=0.642	p<0.001	p<0.001
Glycosylated hemoglobin (%)	5.9±1.2	5.7±1.0	6.3±1.3	5.51±1.1	p<0.001	p=0.218	p<0.001
GFR (ml/min/1.73m2)	48.3±29.8	89.5±17.7	32.6±13.9	–	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 mean±SD, and categorical variables are represented by the number and the percentage of patients. Abbreviations: CONT, control patients; CKD3-5, non-dialysis patients with chronic kidney disease stage 3-5; CKD5D, dialysis patients; GFR, glomerular filtration rate; ACEi, angiotensin-converting enzyme inhibitors; ARB, angiotensin II receptor blockers.

A.II. Baseline results

A.II.a. Detection of circulating ACE2 enzymatic activity in EDTA-plasma samples

EDTA present in the plasma samples binds to the zinc ion, inhibiting the enzymatic activity of ACE2. Therefore, $ZnCl_2$ and $CaCl_2$ were added in excess to the plasma samples to revert the binding of EDTA to the catalytic site of ACE2. Different concentrations of $ZnCl_2$ were tested aned th optimal concentration found for the determination of enzymatic ACE2 activity was 0.5mM (Figure 22A). To confirm that a correct detection of ACE2 activity was achieved by the addition of 0.5 mM of $ZnCl_2$ to the EDTA-plasma samples, recombinant human ACE2 (rhACE2) was added to the samples. The addition of rhACE2 completely inhibited ACE2 activity due to the EDTA present in the samples. As expected, once $ZnCl_2$ was added to the EDTA-plasma samples containing rhACE2, circulating ACE2 activity was restored (Figure 22B).
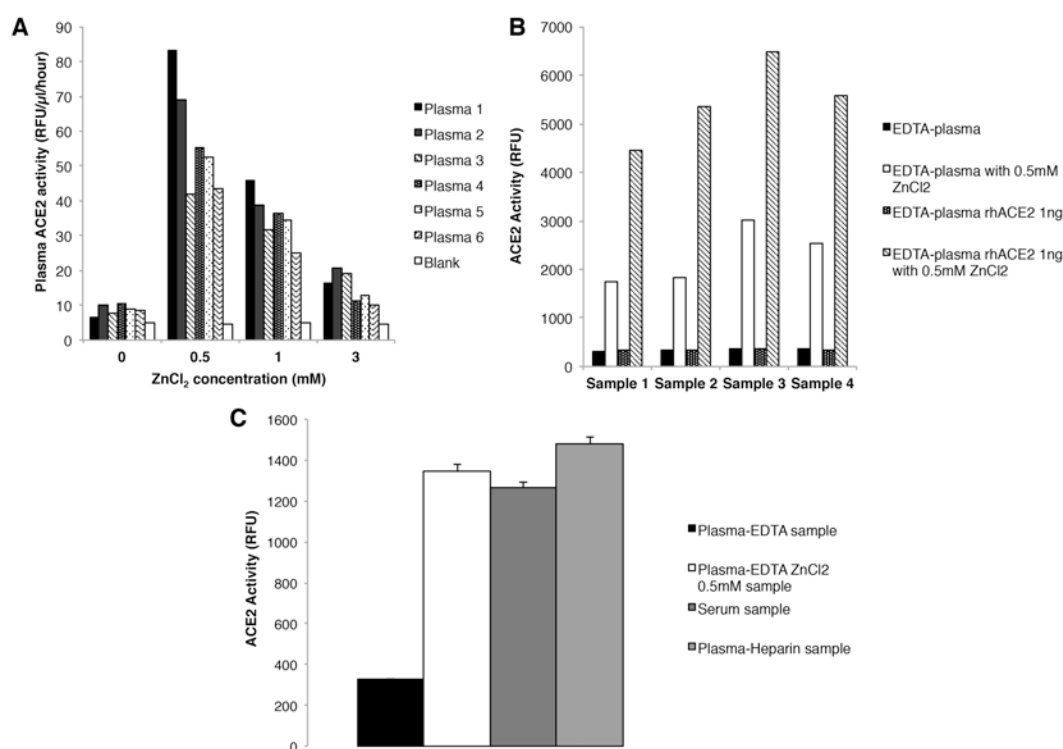
Figure 22. Detection of circulating ACE2 activity in human samples by addition of zinc chloride ($ZnCl_2$). (A) Circulating ACE2 enzymatic activity at increasing concentrations of zinc chloride ($ZnCl_2$). **(B)** ACE2 activity in EDTA-plasma samples with the recombinant human ACE2 (rhACE2) and $ZnCl_2$. **(C)** Mean ACE2 activity in EDTA-plasma samples with and without the rhACE2, serum samples and plasma-heparin samples.

A concentration of 10mM of $CaCl_2$ was also tested to revert the binding of EDTA to the plasma samples, but ACE2 activity in plasma-EDTA samples did not achieve the levels of ACE2 detected in serum (Figure 23). Thus, we used a concentration of 0.5 mM of $ZnCl_2$ for the detection of circulating ACE2 activity.
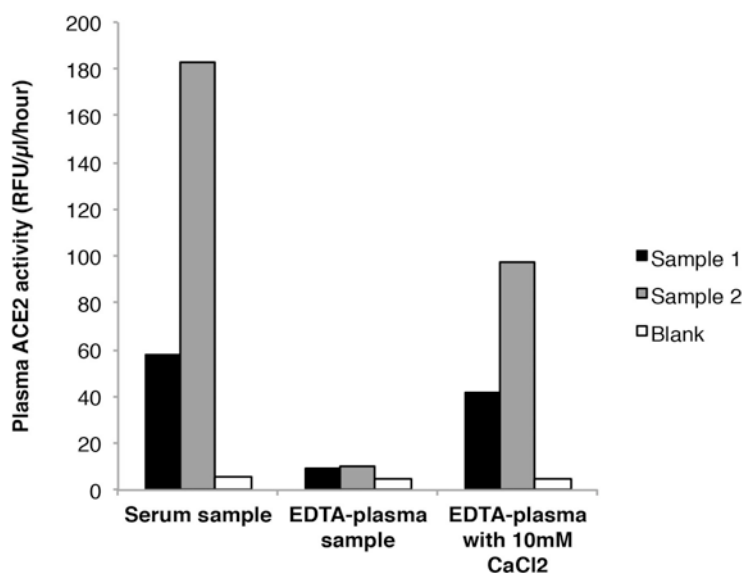
Figure 23. Circulating ACE2 enzymatic activity detection in serum sample, EDTA-plasma sample and EDTA-plasma with 10mM of calcium chloride ($CaCl_2$).

In a set of experiments, serum, plasma-heparin and EDTA-plasma samples were collected from 21 volunteer subjects (Hospital del Mar-IMIM, Barcelona) (Figure 22C). A strong correlation of ≥ 0.95 was found between the different studied samples (EDTA-plasma+$ZnCl_2$, serum and plasma-heparin). In addition, when $ZnCl_2$ was added to the EDTA-plasma, $\geq 91\%$ of ACE2 activity was recovered (Table 19).

Table 19. Intraclass correlation coefficients (ICCs) and percentage of recovery for ACE2 activity between different sample conditions.

ICC (95% Confidence Interval)	
EDTA-plasma ZnCl$_2$ sample versus serum sample	0.95 (0.91-0.97)
Serum sample versus plasma-heparin sample	0.95 (0.91-0.98)
EDTA-plasma ZnCl$_2$ sample versus plasma-heparin sample	0.97 (0.94-0.98)
%Recovery	
EDTA-plasma ZnCl$_2$ sample versus serum sample	107%
EDTA-plasma ZnCl$_2$ sample versus plasma-heparin sample	91%

EDTA-plasma samples from CKD5D patients were obtained before the second dialysis session of the week. To verify that ACE2 is not removed by dialysis, pre- and post-dialysis samples were obtained and serum and EDTA-plasma ACE2 activity was performed (Figure 24). Results indicated that ACE2 is not removed by dialysis, since same values of enzymatic activity are obtained both in serum and EDTA-plasma samples with $ZnCl_2$.
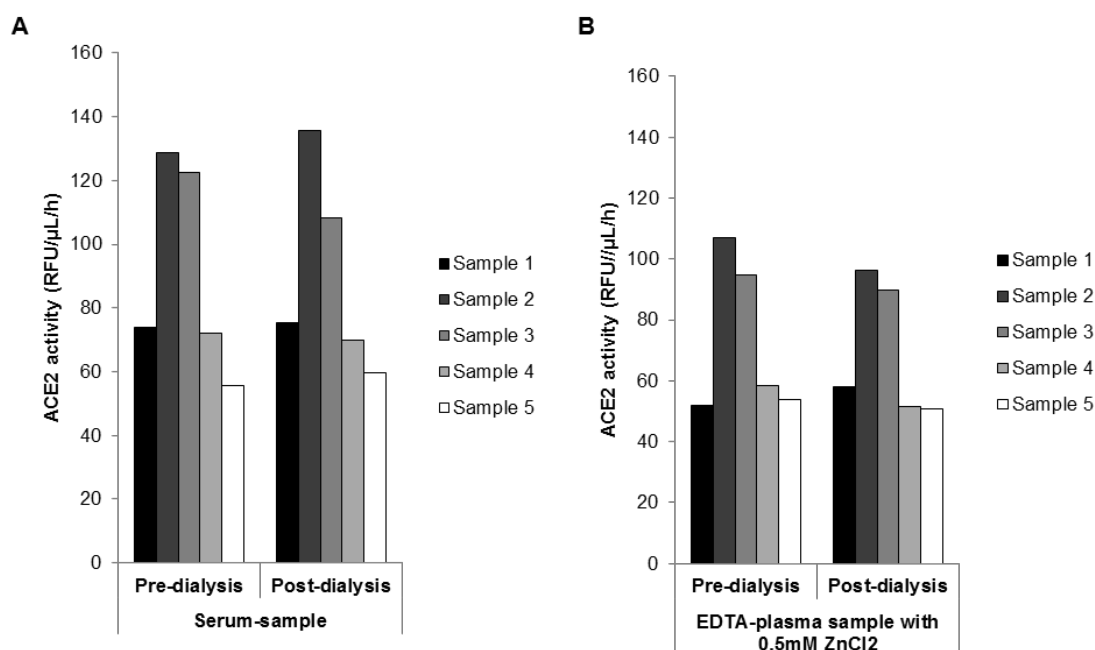
Figure 24. Detection of circulating ACE2 activity in pre- and post-dialysis samples. (A) Circulating ACE2 enzymatic activity in serum from pre- and post-dialysis samples. **(B)** Circulating ACE2 activity in pre- and post-dialysis EDTA-plasma with 0.5mM $ZnCl_2$.

A.II.b. Circulating ACE2 enzymatic activity

Circulating ACE2 activity was significantly decreased in CKD3-5 as compared with CONT patients (45.4 ± 1.12 versus 52.9 ± 1.50 RFU/μL/h, P<0.001) and in CKD5D as compared with CONT (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 25A).

Given that there was a different distribution of age, body weight and prevalence of diabetes, hypertension and dyslipidemia between CONT and CKD patients and also between CKD3-5 and CKD5D subjects (Table 18), paired case-control studies were performed. No differences between CKD3-5 and CKD5D were found (P=0.27) (Figure 25B). Therefore, all groups were analyzed separately.
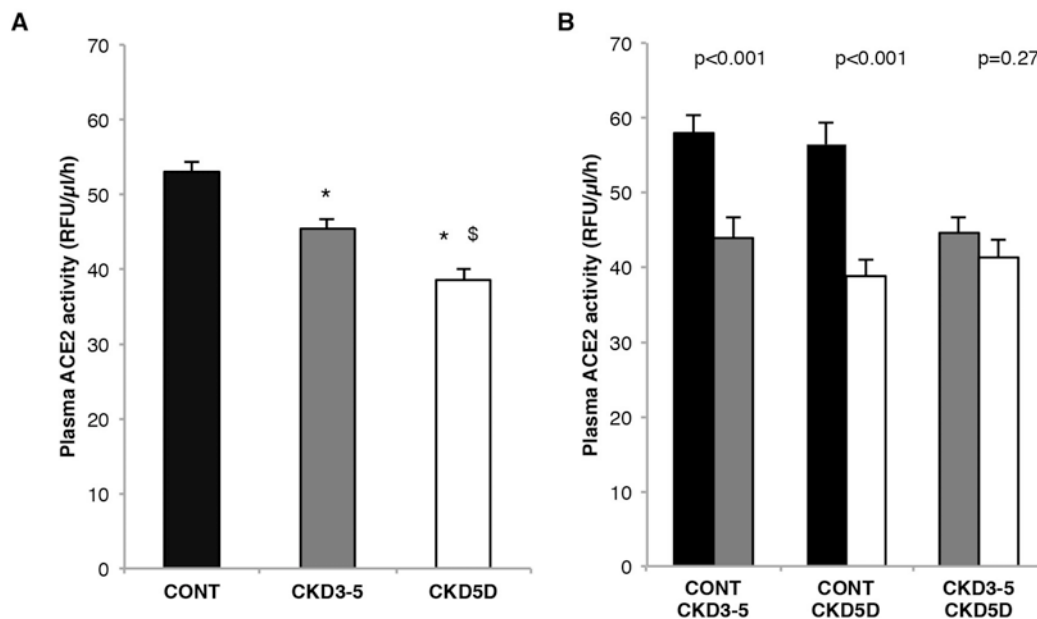
Figure 25. Circulating ACE2 activity in studied subjects. (A) Circulating ACE2 activity between studied groups. **(B)** Circulating ACE2 activity after matching subjects with an equal distribution of gender, diabetes, hypertension, dyslipidemia, smoking habits, weight and age. *P<0.05 versus CONT, $P<0.05 versus CKD3-5.

Table 20 shows the influence of clinical variables and treatments on circulating ACE2 activity. As it is shown, males had significantly increased ACE2 activity when compared with females in all studied groups (P<0.001). Furthermore, patients with plaques had also increased ACE2 activity compared to those without plaques.

Diabetic CONT and CKD3-5 patients 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). 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 patients (P=0.03). A significant direct correlation between ACE2, age and glycosylated hemoglobin in both CONT (P<0.001) and CKD3-5 (P<0.05) was also found. In addition, in CKD5D patients, a direct correlation between ACE2 and age was found (P=0.038).

Circulating ACE2 activity was significantly increased in CONT and CKD5D on ARBs therapy as compared with non-treated patients (P=0.002). Treatment with ACE inhibitors had no influence on circulating ACE2. ACE2 activity was also increased in CONT (P=0.007) and CKD3-5 (P<0.001) under oral antidiabetic agents as compared with non-treated patients. In addition, insulin therapy increased ACE2 activity in CKD3-5 (P<0.001). Circulating ACE2 decreased in CKD5D treated with the vitamin D analog cholecalciferol as compared with non-treated patients (P=0.027).

Table 20. Influence of clinical variables and treatments on circulating ACE2 activity in each studied group.

		Circulating ACE2 activity (RFU/µl/h)±SEM CLINICAL VARIABLES		
		CONT	**CKD3-5**	**CKD5D**
Gender	**Male**	61.4±2.3	50.6±1.5	45.6±2.5
	Female	42.5±1.6*	36.7±1.5*	27.7±1.4*
Diabetes	**No**	51.8±1.6	43.4±1.4	37.1±1.4
	Yes	62.0±4.3*	50.3±2.0*	45.0±6.1
Hypertension	**No**	47.2±1.3	43.2±2.9	36.7±4.1
	Yes	62.0±3.2*	45.6±1.2	38.8±1.8
Dyslipidemia	**No**	49.5±1.6	43.6±1.6	33.9±1.3
	Yes	59.0±3.0*	46.2±1.5	42.7±2.8*
Smoking	**No**	51.7±1.5	44.5±1.2	38.0±1.7
	Yes	57.8±4.4	49.4±3.0*	40.5±4.0
Atherosclerotic plaques	**Absence**	48.9±2.3	39.7±1.9	31.2±1.7
	Presence	56.7±1.9*	48.0±1.4*	41.8±2.2*
		TREATMENTS		
ACEi	**No**	52.5±1.6	46.2±1.5	38.9±1.9
	Yes	57.4±4.3	44.0±1.7	37.2±2.4
ARB	**No**	49.7±1.3	43.7±1.5	37.7±2.2
	Yes	64.2±4.9*	46.7±1.6	40.3±2.0*
Oral antidiabetic drugs	**No**	51.9±1.5	44.2±1.2	38.6±1.6
	Yes	63.1±4.9*	54.6±3.3*	35.1±5.9
Insulin	**No**	52.9±1.5	44.7±1.3	37.3±1.5
	Yes	59.6±11.9	48.9±2.2*	45.5±6.8
Cholecalciferol	**No**	53.0±1.5	45.6±1.2	39.0±1.7
	Yes	56.9±0.0	37.9±4.5	25.0±3.9*

Abbreviations: SEM, standard error mean; 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).

Multivariate linear regression analyses were performed to identify independent predictors of circulating ACE2 activity in the studied groups (Table 21). Male gender and advanced age were independent predictors of ACE2 in all studied groups. Diabetes was also associated with increased ACE2 activity in CKD3-5. In addition, ARBs and cholecalciferol therapies were independent predictors of ACE2 in CKD5D.

Table 21. Multiple linear regression analysis of potential predictors of circulating ACE2 activity.

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

Data are expressed as regression coefficients and p values. Dependent variable: circulating ACE2 activity, expressed in LnACE2. Abbreviations: CONT, control patients; CKD3-5, non-dialysis patients with chronic kidney disease stage 3-5; CKD5D, dialysis patients; ARB, angiotensin II receptor blockers.

A.II.c. Detection of circulating ACE enzymatic activity in EDTA-plasma samples

Given that EDTA in the plasma samples also binds to the zinc-binding site of ACE, different concentrations of $ZnCl_2$ and $CaCl_2$ were tested. For the determination of ACE activity, an optimal concentration of 15.63 mM of $ZnCl_2$ was found (Figure 26).
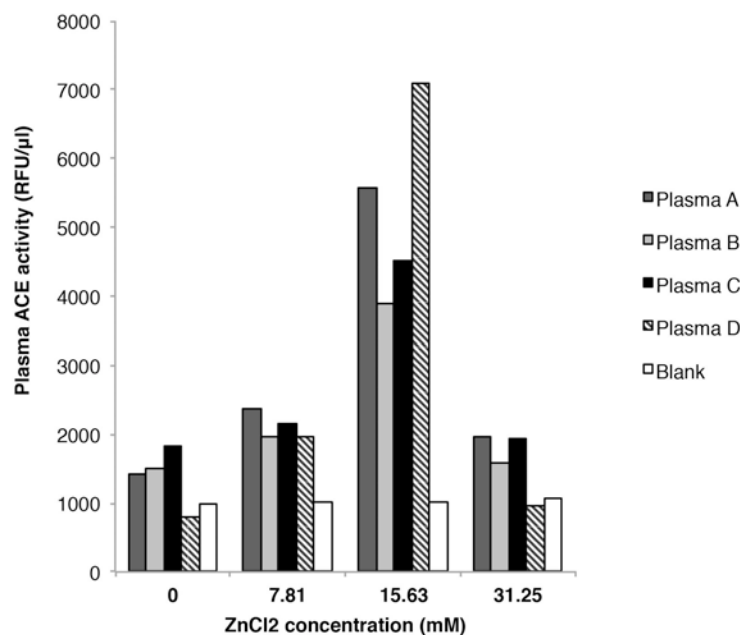
Figure 26. Circulating ACE enzymatic activity at increased concentrations of zinc chloride (ZnCl$_2$).

As for ACE2 activity, CaCl$_2$ to disinhibit the effect of EDTA on ACE activity was also tested at a concentration of 10 mM. CaCl$_2$ allowed the detection of ACE activity but it did not achieve the levels observed in serum samples (Figure 27A). In addition, different concentrations of CaCl$_2$ were tested and compared with the 15.63 mM concentration of ZnCl$_2$, previously reported to be the optimal concentration for ACE detection (Figure 27B). This analysis confirmed that CaCl$_2$ was capable of reverting EDTA binding to the zinc-binding site of ACE but it was not optimal for the detection of circulating ACE activity.
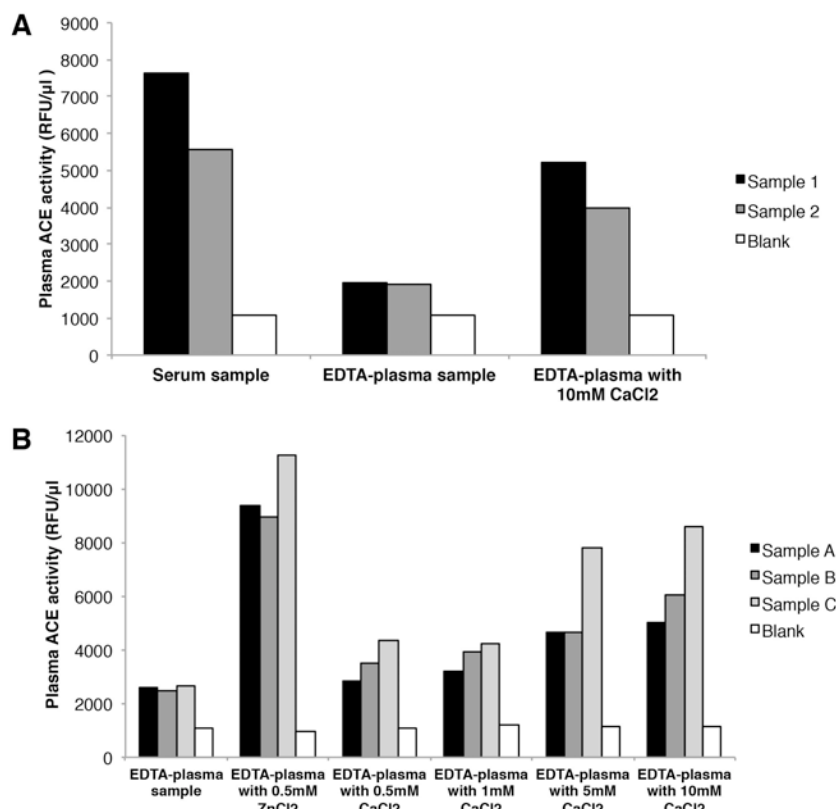
Figure 27. Detection of circulating ACE activity by addition of calcium chloride (CaCl$_2$) **(A)** Circulating ACE enzymatic activity detection in serum sample, EDTA-plasma sample and EDTA-plasma with 10mM of CaCl$_2$. **(B)** Circulating ACE enzymatic activity detection at increased concentration of CaCl$_2$ compared to the optimal concentration of zinc chloride (ZnCl$_2$).

As it was performed for the ACE2 activity, ACE activity was calculated in EDTA-plasma, serum and plasma-heparin samples from 21 subjects. A strong correlation of ≥ 0.84 was found between the different studied samples (EDTA-plasma+ZnCl$_2$, serum and heparin). In addition, when ZnCl$_2$ was added to the EDTA-plasma, $\geq 83\%$ of ACE activity was recovered (Table 22).

Table 22. Intraclass correlation coefficients (ICCs) and percentage of recovery for ACE activity between different sample conditions.

ICC (95% Confidence Interval)	
EDTA-plasma ZnCl$_2$ sample versus serum sample	0.90 (0.90-0.95)
Serum sample versus plasma-heparin sample	0.93 (0.86-0.97)
EDTA-plasma ZnCl$_2$ sample versus plasma-heparin sample	0.84 (0.68-0.92)
%Recovery	
EDTA-plasma ZnCl$_2$ sample versus serum sample	89%
EDTA-plasma ZnCl$_2$ sample versus plasma-heparin sample	83%

As for the ACE2 activity, ACE activity was also performed in pre- and post-dialysis serum and EDTA-plasma samples (Figure 28), confirming that ACE was not removed by dialysis and could be detected in CKD5D patients.
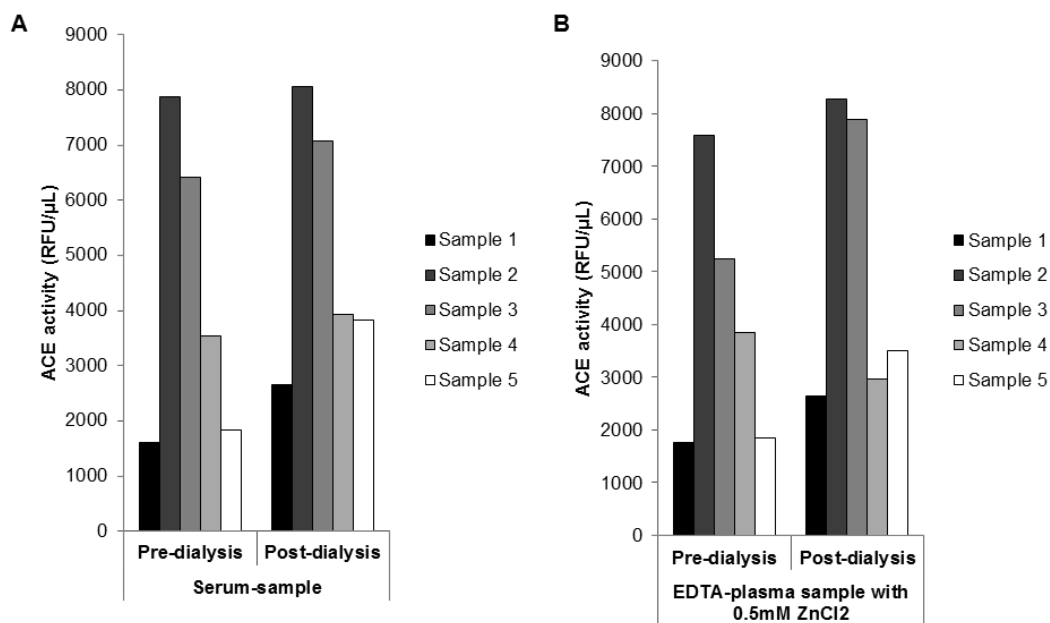
Figure 28. Detection of circulating ACE activity in pre- and post-dialysis samples. (A) Circulating ACE enzymatic activity in serum from pre- and post-dialysis samples. **(B)** Circulating ACE activity in pre- and post-dialysis EDTA-plasma with 0.5mM ZnCl$_2$.

A.II.d. Circulating ACE enzymatic activity

Circulating ACE activity was significantly increased in CKD3-5 (4181 ± 58.37 versus 3809 ± 71.96 RFU/µL, P=0.035) and in CKD5D as compared with CONT (4454 ± 87.10 versus 3809 ± 71.96, P<0.001). In addition, ACE activity was significantly increased in CKD5D as compared with CKD3-5 (4454 ± 87.10 versus 4181 ± 58.37, P=0.001) (Figure 29A).

As it was performed for ACE2 activity and given the differences in patients' characteristics, paired case-control studies were performed. Results showed that circulating ACE activity was increased in CKD3-5 and CKD5D as compared with CONT; and it was also increased in CKD5D as compared with CKD3-5 (Figure 29B).
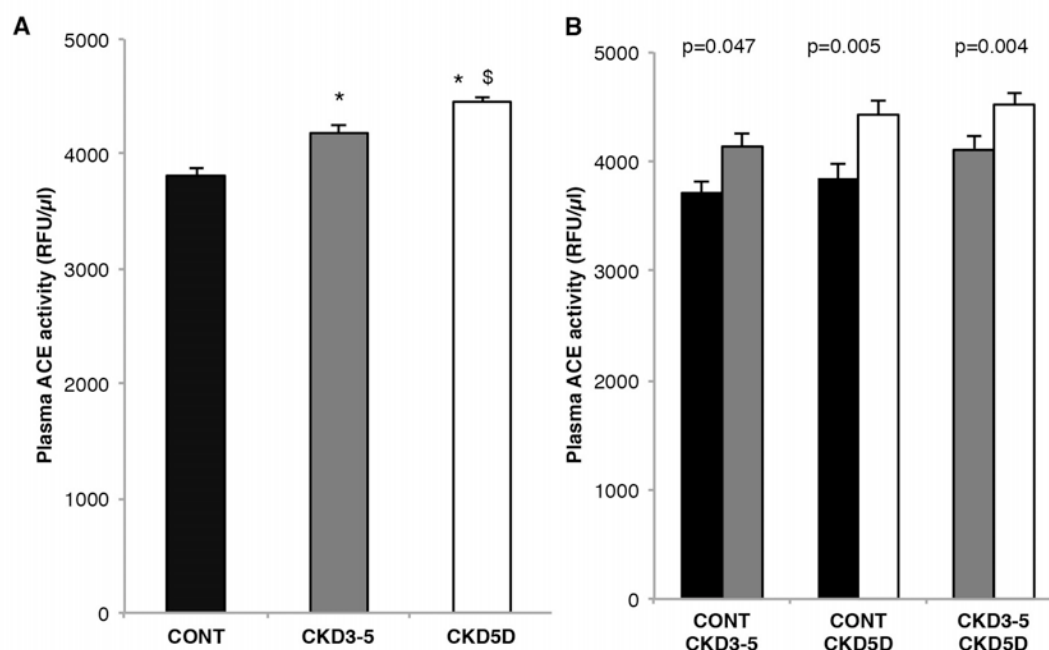
Figure 29. Circulating ACE activity in studied subjects. (A) Circulating ACE activity between studied groups. **(B)** Circulating ACE activity after matching subjects with an equal distribution of gender, diabetes, hypertension, dyslipidemia, smoking habits, weight and age. *P<0.05 versus CONT, $P<0.05 versus CKD3-5.

Analyses of the influence of clinical variables on circulating ACE activity (Table 23) showed that females had increased ACE activity as compared with males in CONT and CKD3-5 (P<0.001). However, no differences were observed in CKD5D patients (P=0.057). CONT patients with plaques showed decreased levels of circulating ACE activity as compared with those patients without plaques (P=0.011), but no differences were found in CKD3-5 and CKD5D patients. ACE activity was decreased in CKD3-5 patients with hypertension or dyslipidemia as compared with non-hypertensive (P=0.001) or without dyslipidemia (P=0.004). A significant indirect correlation was found between circulating ACE activity, age (P=0.033) and glycosylated hemoglobin (P=0.019) in CONT subjects.

Regarding treatments (Table 23), increased ACE activity was found in CONT and CKD3-5 in ARBs therapy as compared with non-treated patients (P=0.023 and P<0.001, respectively). As expected, subjects treated with ACE inhibitors had lower levels of ACE activity as compared with non-treated subjects in all groups (P<0.001 for all groups). ACE activity decreased in CKD3-5 and CKD5D treated with cholecalciferol as compared with non-treated (P=0.002 and P=0.015, respectively).

Table 23. Influence of clinical variables and treatments on circulating ACE activity in each studied group.

		Circulating ACE activity (RFU/µl)±SEM CLINICAL VARIABLES		
		CONT	**CKD3-5**	**CKD5D**
Gender	**Male**	3541±94.8	4032±71.8	4575±109.9
	Female	4144±106.9*	4426±98.6*	4270±141.8
Diabetes	**No**	3766±75.4	4103±66.7	4460±97.2
	Yes	4127±230.3	4378±117.7	4431±196.3
Hypertension	**No**	3825±89.6	4669±189.6	14666±188.5
	Yes	3782±120.5	4130±61.2*	4418±96.8
Dyslipidemia	**No**	3806±90.0	4404±105.8	4370±118.1
	Yes	3814±120.1	4080±69.8*	4531±127.0
Smoking	**No**	3817±80.7	4229±65.2	4414±94.4
	Yes	3775±159.7	3977±130.5	4600±212.3
Atherosclerotic plaques	**Absence**	3995±106.0	4160±99.9	4310±157.8
	Presence	3638±97.0*	4192±71.8	4518±104.4
		TREATMENTS		
ACEi	**No**	3908±75.5	4994±70.3	4946±87.5
	Yes	2890±203.0*	2695±64.6*	2410±145.4*
ARB	**No**	3720±80.3	3730±85.2	4367±102.4
	Yes	4116±158.3*	4535±77.8*	4647±163.8
Oral antidiabetic drugs	**No**	3795±75.2	4165±61.9	4465±88.1
	Yes	3938±245.2	4308±175.2	3954±582.9
Insulin	**No**	3805±72.1	4113±61.6	4471±94.4
	Yes	4087±848.3	4550±168.3	4357±227.5
Cholecalciferol	**No**	3805±72.0	4214±59.7	4493±88.3
	Yes	6215±0.0	3211±229.2*	3251±438.7*

Abbreviations: SEM, standard error mean; 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).

Multivariate linear regression analyses were also performed to identify independent predictors of circulating ACE activity in the studied groups (Table 24). As well as in the bivariate analysis, ACE inhibitor therapy was inversely associated with ACE activity in all studied groups. In CONT, female gender, younger age and ARB treatment were also identified as independent predictors of circulating ACE activity. In CKD3-5 patients, female gender, younger age and diabetes were found as a potential independent predictor of circulating ACE activity. Cholecalciferol treatment was found to be associated with ACE activity in CKD5D.

Table 24. Multiple linear regression analysis of potential predictors of circulating ACE activity.

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 variable: circulating ACE activity, expressed in LnACE. Abbreviations: 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.

A.III. 2-year follow-up results

For the prospective study at 2-years of follow-up, a total of 1548 CKD3-5 patients from the baseline study were included.

A.III.a. Baseline 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, $p=0.154$) (Figure 30A). There were no

differences in baseline circulating ACE2 activity between patients with a decrease in 50% of estimated GFR and stable patients (52.17±6.86 versus 43.01±1.28, p=0.132) (Figure 30B). In addition, 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) (Figure 30C-D).
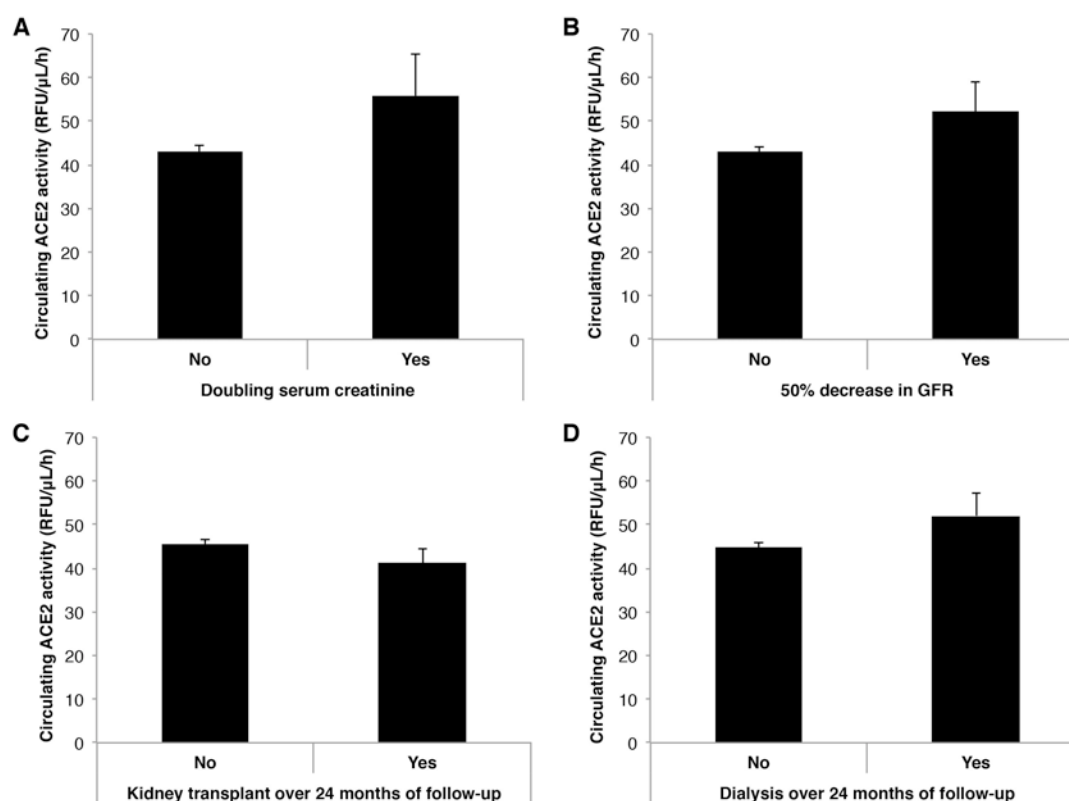
Figure 30. Association of renal function with baseline circulating ACE2 activity: (A) doubling of serum creatinine; **(B)** reduction of GFR; **(C)** Kidney transplant over 24 months of follow-up; **(C)** Dialysis over 24 months of follow-up. Abbreviations: GFR, glomerular filtration rate.

A.III.b. Baseline ACE2 activity and atherosclerosis

The relationship between baseline circulating ACE2 and the evolution of plaques was studied in CKD3-5 patients. Regarding general plaque (carotid and/or femoral), patients with *de novo* plaque (41.97±2.44) and baseline plaque (48.14±1.71) showed significantly higher levels of circulating ACE2 as compared with patients with no plaque (34.04±1.55, P=0.007 and P<0.001, respectively) (Figure 31A). Same pattern was observed when femoral plaques were analyzed independently: circulating ACE2 activity was increased in patients with *de novo* (46.62±3.19) and baseline (48.85±1.90) femoral plaque as compared to those patients with no plaque (neither at baseline nor at 2-years of follow-up) (35.89±1.50, P=0.002 and P<0.001, respectively) (Figure

31B). 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 31C).
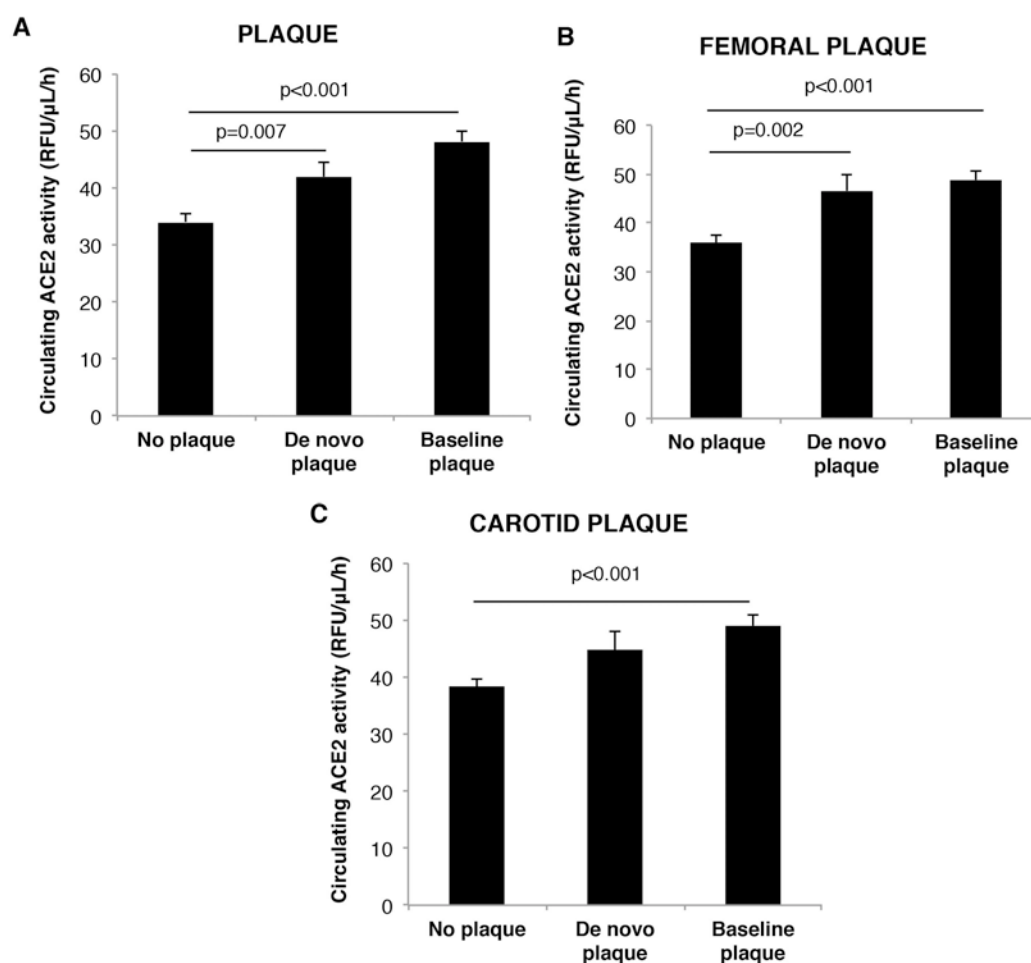
Figure 31. Baseline circulating ACE2 and evolution of plaques over 2-years of follow-up. (A) General plaque (carotid and/or femoral). **(B)** Femoral plaque. **(C)** Carotid plaque.

The levels of baseline circulating ACE2 activity were also determined according to the number of territories with plaques (maximum of 10 territories) at 24 months (Figure 32A). Patients with 1-4 or ≥5 number of territories with plaques (43.02±1.48 and 52.07±2.96, respectively) showed increased levels of baseline ACE2 activity as compared to patients without plaque at 24 months of follow-up (34.50±1.80, p<0.001 and p<0.001, respectively). In addition, baseline circulating ACE2 activity was significantly increased in patients with ≥5 territories with plaques as compared with patients with 1-4 territories (P=0.003) (Figure 32A). The increase in the number of territories from baseline to 24-months of follow-up was also assessed and association with ACE2 activity showed that patients with an increase in the number of territories with plaques over 2 years of follow-up had higher levels of baseline ACE2 activity as compared with stable patients (46.90±1.63 versus 38.73±1.59, p<0.001) (Figure 32B).
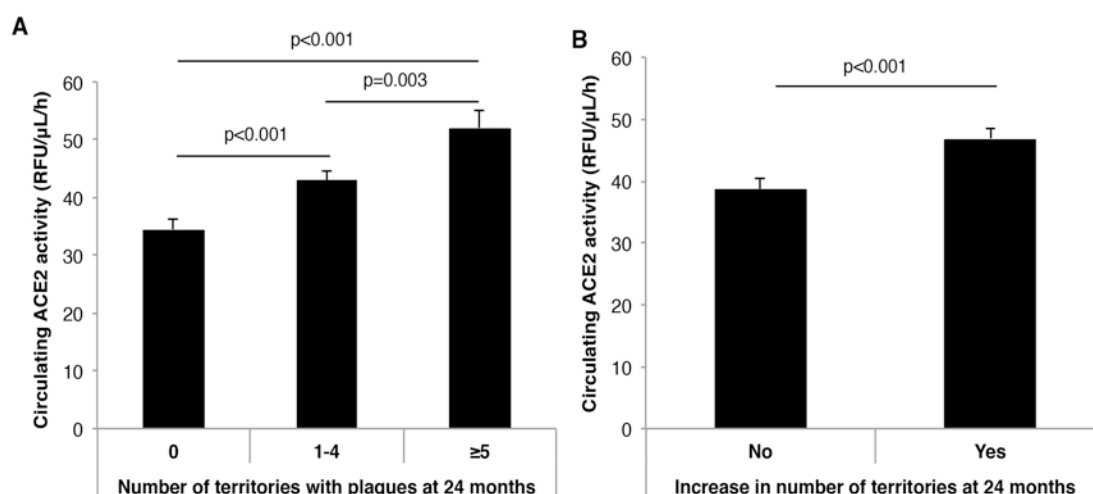
Figure 32. Association of baseline circulating ACE2 with **(A)** the number of territories with plaques, classified in three groups (0, 1-4 and ≥5) and **(B)** the increase in number of territories with plaques at 24 months.

Clinical data according the number of territories with plaques at 24 months are shown in Table 25. 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 CV disease was also increased with the number of territories with plaques, but it did not reach statistical significance. There were no differences between renal function and the number of territories with plaques. Regarding the increase in number of territories with plaques, the same pattern as with the number of territories with plaques was observed (Table 26).

Table 25. Clinical parameters according the number of territories with plaques at 24-months of follow-up.

	Number of territories with plaques			
	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 CV disease (%)	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 GFR (%)	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

Values for categorical variables are given as percentage; values for continuous variable as mean ±SD. CV: cardiovascular; GFR: glomerular filtration rate.

Table 26. Clinical parameters according the increase in number of territories with plaques over 24-months of follow-up.

	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 CV disease (%)	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. CV: cardiovascular; GFR: glomerular filtration rate.

Other known markers of atherosclerosis are IMT, ABI and AD. We found a significant but weak correlation between baseline ACE2 activity and IMT at 24 months (r=0.07, p=0.023). Furthermore, baseline ACE2 activity was higher in patients with pathological ABI (defined as a value ≤0.9 and ≥1.4) as compared to patients with normal ABI at 24-months of follow-up (51.53±3.49 versus 42.13±1.20, p=0.002) (Figure 33A). Patients with severe AD (AD 2-3) showed increased levels of baseline circulating ACE2 activity as compared with those patients with incipient AD (AD 0-1) (47.47±1.58 versus 35.74±1.83, p<0.001) (Figure 33B).
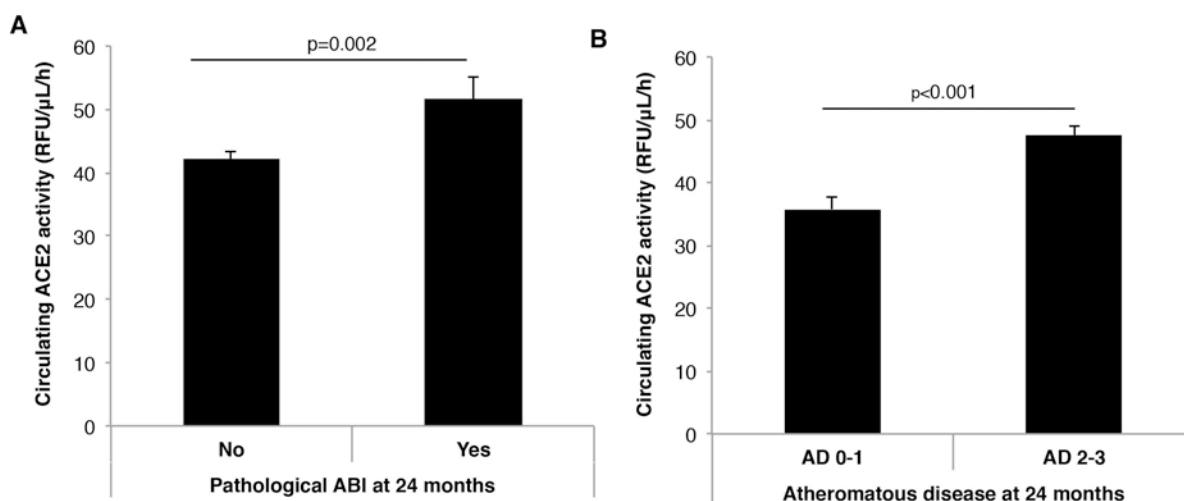
Figure 33. Association of subclinical atherosclerosis with baseline circulating ACE2 activity. (A) Pathological ABI at 24 months of follow-up and **(B)** Atheromatous disease at 24-months of follow-up.

Multivariate linear regression analyses were performed (Table 27). In the first model, baseline circulating ACE2 activity was the dependent variable and the model was adjusted by age, gender and diabetes. Pathological ABI and increased number of territories with plaques were independently associated with increased baseline circulating ACE2 activity (Table 27, Model 1). To confirm the role of baseline ACE2 activity as a potential biomarker of atherosclerosis at 24 months of follow-up, a second model with the number of territories with plaques at 24 months as dependent variable was performed. The analysis was also adjusted by age, gender and diabetes. In this second model, male gender, older age, diabetes and increased baseline circulating ACE2 activity were potential independent predictors of atherosclerosis at 2 years of follow-up (Table 27, Model 2).

Table 27. Multivariate linear regression analyses.

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

Data are expressed as regression coefficients and P-value. Model 1: multivariate linear regression analysis of potential predictors of baseline circulating ACE2 (R=0.301); Dependent variable: baseline circulating ACE2 activity (expressed in LnACE2). Model 2: multivariate linear regression analysis of potential predictors of territories with plaques at 24-months of follow-up (R=0.559); Dependent variable: number of territories with plaques at 24 months. Abbreviations: ABI, ankle-brachial index.

The ROC curve, generated by logistic regression with patients having territories with plaques at 24 months of follow-up as the dependent variable, showed baseline circulating ACE2 activity as a potential predictor of the number of territories with plaques (ROC area under the ROC curve = 0.614, 95% CI: 0.571-0.657) (Figure 34). An optimal cut-off value of 24.935 RFU/μL/h was found with sensitivity of 0.717 and specificity of 0.462.
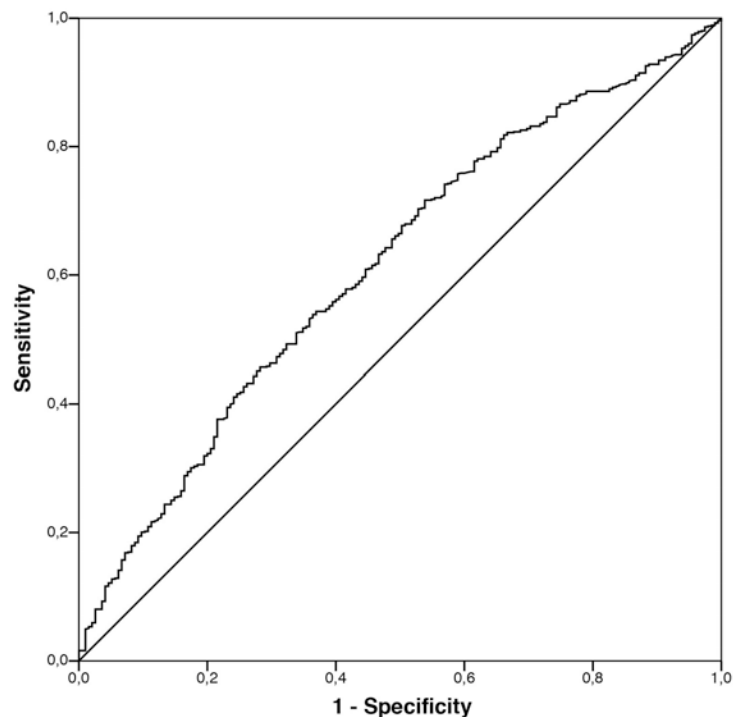
Figure 34. ROC curve analysis. ROC curve for territories with plaques at 24 months of follow-up and baseline circulating ACE2 activity, AUC 0.614 (0.571-0.657, p<0.001).

A.III.c. Baseline ACE2 activity and events

No differences were found regarding baseline circulating ACE2 activity, CV events and CV mortality during the 2-years of follow-up. However, baseline circulating ACE2 was significantly higher in patients with non-CV and all-cause mortality (Table 28).

Table 28. Association of events and mortality with baseline circulating ACE2 activity.

		Baseline circulating ACE2 activity	P-value
CV event	**No**	45.40±1.16	0.167
	Yes	43.63±3.33	
CV mortality	**No**	45.40±1.13	0.705
	Yes	38.88±4.90	
Non-CV 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 ± SEM

A.IV. 4-year follow-up analysis

For the prospective study at 4 years of follow-up, a total of 1237 CKD3-5 patients from the baseline study were included.

The univariate association between baseline circulating ACE2 and 4-year follow-up events was analyzed (Figure 35). Patients with non-fatal and fatal CV event at 48 months showed higher baseline circulating ACE2 activity as compared to patients without any event during the follow-up (46.58±2.94 versus 43.03±1.12, p=0.010) (Figure 35A). In addition, baseline ACE2 activity was also increased in patients with non-CV mortality (62.43±8.74) and all-cause mortality (55.42±5.77) (Figure 35B and D) as compared to patients that suffered no events (43.03±1.12, p=0.018 and p=0.006, respectively). When all-cause mortality and non-fatal CV events patients were analyzed together, baseline circulating ACE2 activity was also found increased as compared to patients without any event (52.15±3.65 versus 43.03±1.12, p=0.001) (Figure 35C).
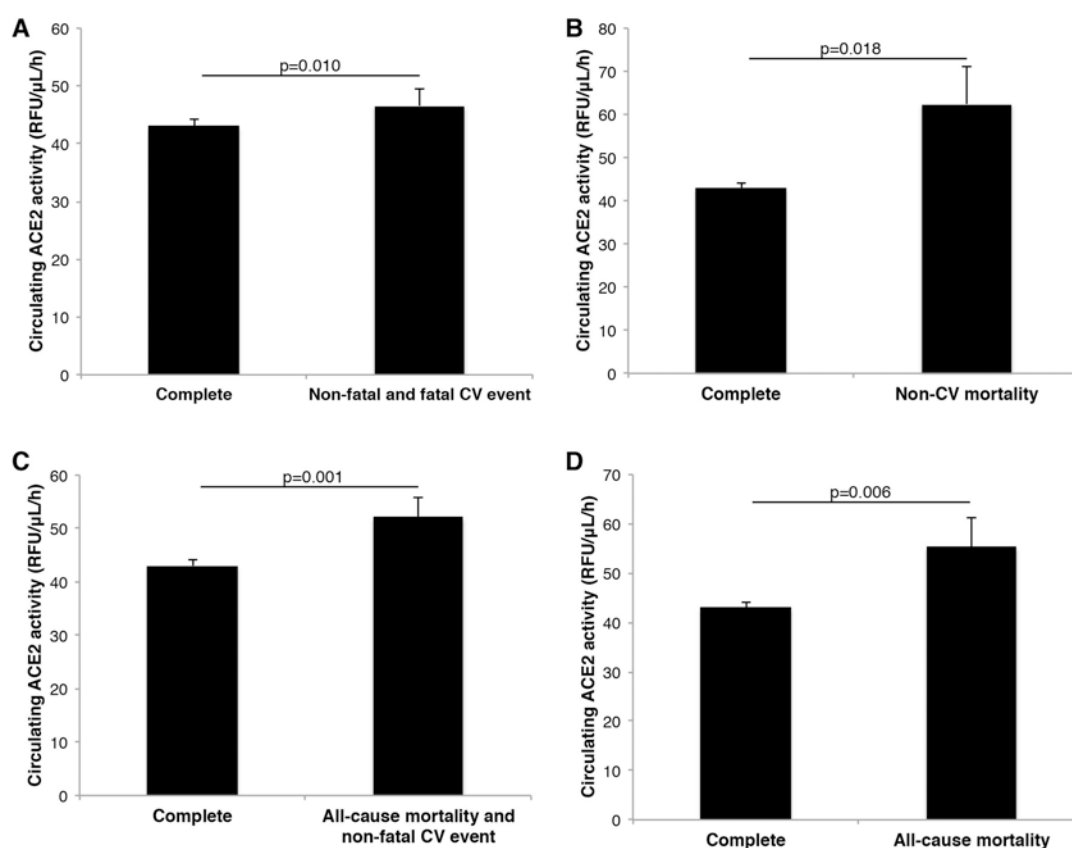
Figure 35. Association of 4-year follow-up events with baseline circulating ACE2 activity. (A) Non-fatal and fatal CV event; **(B)** Non-CV mortality; **(C)** All-cause mortality and non-fatal CV event; and **(D)** All-cause mortality. Complete refer to patients that have ended follow-up without any event or loss due to kidney transplantation or loss of follow-up.

For the overall survival analysis, stratified baseline circulating ACE2 activity according to the cut-off value (low-level ACE2: <24.9 RFU/μL/h and high-level ACE2: ≥24.9 RFU/μL/h) obtained in the ROC curve analysis at 2 years of follow-up was used. Estimated mortality in patients with non-fatal and fatal CV events was higher in the high-level ACE2 group (9.4%) as compared to the low-level group (5.1%). Using the log-rank test to compare these two Kaplan-Meier survival curves, significant difference was found (p=0.008) (Figure 36A). No differences were observed for the estimated non-CV mortality between groups (3.8% for the low-level group and 5.3% for the low-level group, p=0.386) (Figure 36B). Estimated mortality was also assessed in patients with the composite all-cause mortality and non-fatal CV event and in patients with all-cause mortality. In both cases, estimated mortality was higher in the high-level ACE2 group (14.1% and 8.5%, respectively) as compared to the low-level ACE2 group (8.7% and 4.8%, respectively; p=0.008 and p=0.024, respectively) (Figure 36C-D).
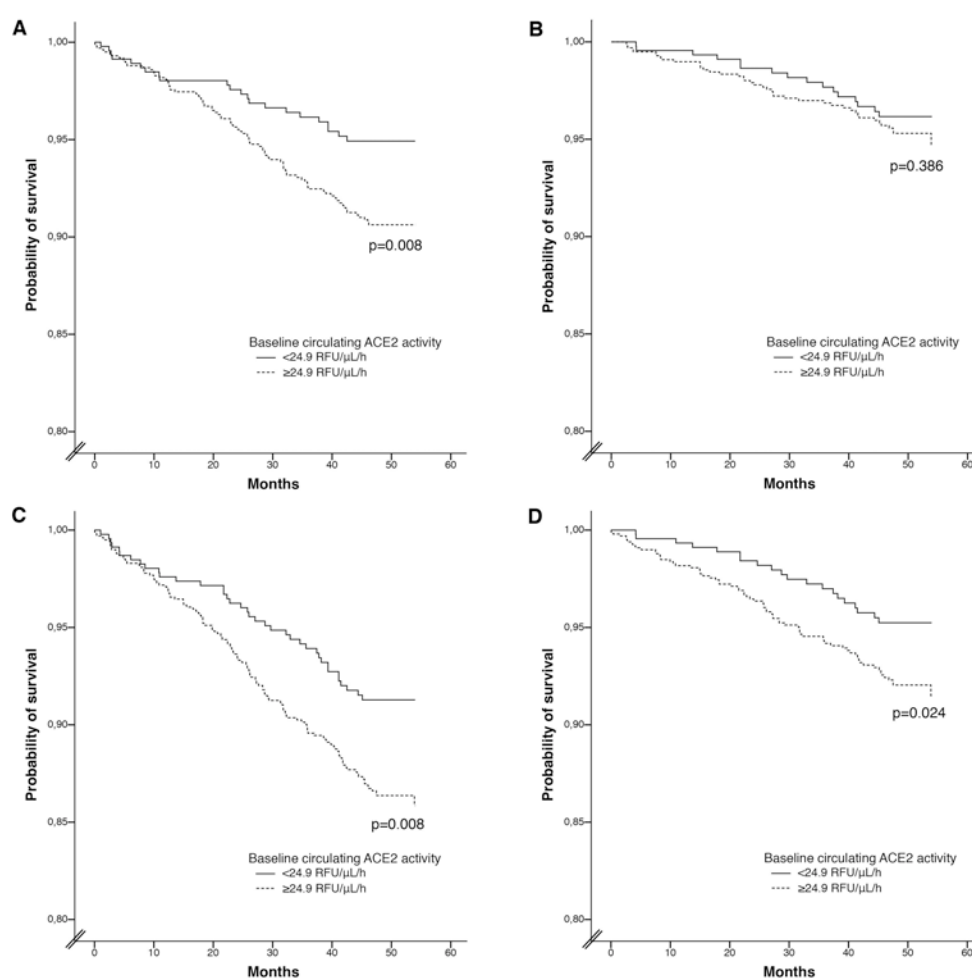
Figure 36. Kaplan-Meier survival analyses. (A) Non-fatal and fatal CV events; (B) Non-CV mortality, (C) All-cause mortality and non-fatal CV event; and (D) All-cause mortality.

Risk factors of 4-year follow-up events were analyzed in CKD3-5 patients. For the analysis of risk factors of 4-year follow-up events, baseline circulating ACE2 activity was included in the analysis. For non-CV mortality, ACE2 activity was included as a continuous variable, while for the other events analyzed (non-fatal and fatal CV event; all-cause mortality and non-fatal CV event; and all-cause mortality) baseline ACE2 activity was stratified according to the cut-off value (24.9 RFU/μL/h) obtained in the ROC curve analysis at 2 years of follow-up. Given that the cut-off value was obtained as a result of the association between the number of territories with plaques and baseline circulating ACE2 activity, this value was only used for those events implying a CV outcome, but not for non-CV mortality.

Univariate analysis showed that advanced age ($\geq$ 65 years), male gender, smoking habits, diabetes, creatinine levels, baseline circulating ACE2 activity ($\geq$ 24.9 RFU/μL/h), SBP, pulse pressure and phosphorous were risk factors for the composite end-point of non-fatal and fatal CV event, for the composite all-cause mortality and non-fatal CV event and for all-cause mortality (Table 29). On the contrary, increased GFR and 25-hydroxi vitamin D were inversely associated with 4-year follow-up events (Table 29). In non-CV mortality, advanced age ($\geq$ 65 years) and increased creatinine and baseline circulating ACE2 activity were determined as risk factors. Increased GFR, total cholesterol and hemoglobin were inversely associated with non-CV mortality (Table 30).

RESULTS

Table 29. Univariate logistic regression to model 4-year follow-up outcomes.

	Non-fatal and fatal CV event		All-cause mortality and non-fatal CV event		All-cause mortality	
	HR (95% CI)	p-value	HR (95% CI)	p-value	HR (95% CI)	p-value
Age, <65 vs ≥65 years	1.72 (1.2-2.6)	0.005	2.24 (1.6-3.1)	<0.001	2.85 (1.8-4.4)	<0.001
Gender, men vs women	1.71 (1.1-2.6)	0.014	1.70 (1.2-24)	0.002	1.81 (1.1-2.9)	0.013
Current smoker, yes vs no	1.56 (1.1-2.3)	0.029	1.59 (1.2-2.2)	0.005	1.57 (1.0-2.4)	0.042
Diabetes, yes vs no	2.56 (1.8-3.7)	<0.001	2.21 (1.6-3.0)	<0.001	2.40 (1.6-3.6)	<0.001
Dyslipidemia, yes vs no	1.84 (1.1-3.0)	0.014	1.32 (0.9-1.9)	0.129	1.07 (0.7-1.7)	0.772
Creatinine, mg/dL	1.19 (1.0-1.4)	0.02	1.20 (1.1-1.4)	0.002	1.24 (1.1-1.4)	0.006
GFR, mL/min/1.73m^2	0.99 (0.9-1.0)	0.07	0.98 (0.9-1.0)	0.003	0.98 (0.9-1.0)	0.003
Baseline circulating ACE2 activity, <24.935 vs ≥24.935 RFU/µL/h	1.87 (1.2-3.0)	0.009	1.63 (1.1-2.3)	0.009	1.76 (1.1-2.9)	0.025
BMI, kg/m^2	1.03 (1.0-1.1)	0.095	1.01 (0.9-1.1)	0.369	1.02 (0.9-1.1)	0.46
SBP, mmHg	1.02 (1.0-1.1)	<0.001	1.01 (1.0-1.1)	<0.001	1.01 (1.0-1.1)	0.02
DBP, mmHg	0.99 (1.0-1.1)	0.829	0.99 (0.9-1.0)	0.549	0.99 (0.9-1.0)	0.508
Pulse pressure, mmHg	1.02 (1.0-1.1)	<0.001	1.02 (1.0-1.1)	<0.001	1.02 (1.0-1.1)	0.002
Glucose, mg/mL	1.00 (1.0-1.1)	0.026	1.00 (0.9-1.0)	0.227	1.00 (0.9-1.0)	0.743
Total cholesterol, mg/dL	1.00 (0.9-1.0)	0.572	0.99 (0.9-1.0)	0.39	0.99 (0.9-1.0)	0.145
HDL cholesterol, mg/dL	0.98 (0.9-1.0)	0.003	0.99 (0.9-1.0)	0.032	0.99 (0.9-1.0)	0.185
LDL cholesterol, mg/dL	1.00 (0.9-1.0)	0.244	1.00 (0.9-1.0)	0.868	1.00 (0.9-1.0)	0.549
Glycosylated hemoglobin, %	1.33 (1.1-1.6)	<0.001	1.17 (1.0-1.4)	0.035	0.94 (0.7-1.2)	0.61
Hemoglobin, g/dL	0.93 (0.8-1.0)	0.925	0.85 (0.8-0.9)	0.001	0.77 (0.7-0.9)	<0.001
Phosphorous, mg/dL	1.28 (1.0-1.6)	0.028	1.24 (1.0-1.5)	0.025	1.29 (1.0-1.6)	0.039
Parathyroid hormone, pg/mL	1.00 (0.9-1.0)	0.284	1.00 (0.9-1.0)	0.266	1.00 (0.9-1.0)	0.395
25-hydroxi vitamin D, ng/mL	0.94 (0.9-1.0)	0.01	0.95 (0.9-1.0)	0.001	0.97 (0.9-1.0)	0.052
1,25-dihydroxi vitamin D, pg/mL	0.96 (0.9-1.0)	0.002	0.97 (0.9-1.0)	0.003	0.98 (0.9-1.0)	0.131
ACEi treatment, yes vs no	1.19 (0.8-1.7)	0.388	1.03 (0.8-1.4)	0.842	1.08 (0.7-1.6)	0.723
ARB treatment, yes vs no	1.52 (1.0-2.3)	0.041	0.99 (0.7-1.4)	0.97	0.57 (0.4-0.9)	0.007
Statin treatment, yes vs no	1.45 (1.0-2.2)	0.082	1.15 (0.8-1.6)	0.398	0.88 (0.6-1.3)	0.535

Results are expressed as hazard ratio (HR) and 95% confidence intervals (95% CI). Abbreviations: CV, cardiovascular; GFR, glomerular filtration rate; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HDL, high-density lipoprotein; LDL, low-density lipoprotein; ACEi, angiotensin-converting enzyme inhibitors; ARB, angiotensin receptor 1 blocker.

Table 30. Univariate logistic regression to model 4-year follow-up non-CV mortality.

	Non-CV mortality	
	HR (95% CI)	p-value
Age, <65 vs ≥65 years	3.91 (2.14-7.13)	<0.001
Gender, men vs women	1.68 (0.94-2.98)	0.079
Current smoker, yes vs no	1.64 (0.95-2.83)	0.078
Diabetes, yes vs no	1.67 (0.98-2.84)	0.058
Dyslipidemia, yes vs no	0.80 (0.47-1.37)	0.419
Creatinine, mg/dL	1.23 (1.01-1.49)	0.038
GFR, mL/min/1.73m^2	0.97 (0.95-0.99)	0.011
Baseline circulating ACE2 activity, RFU/µL/h	1.01 (1.00-1.01)	0.001
BMI, kg/m^2	0.98 (0.93-1.03)	0.452
SBP, mmHg	1.01 (0.99-1.02)	0.377
DBP, mmHg	0.99 (0.97-1.02)	0.473
Pulse pressure, mmHg	1.01 (0.99-1.02)	0.144
Glucose, mg/mL	0.99 (0.98-1.00)	0.323
Total cholesterol, mg/dL	0.99 (0.98-1.00)	0.025
HDL cholesterol, mg/dL	1.00 (0.99-1.02)	0.664
LDL cholesterol, mg/dL	0.99 (0.98-1.00)	0.059
Glycosylated hemoglobin, %	0.76 (0.52-1.11)	0.159
Hemoglobin, g/dL	0.73 (0.61-0.86)	<0.001
Phosphorous, mg/dL	1.14 (0.82-1.58)	0.427
Parathyroid hormone, pg/mL	1.00 (0.99-1.00)	0.672
25-hydroxi vitamin D, ng/mL	0.98 (0.94-1.02)	0.23
1,25-dihydroxi vitamin D, pg/mL	0.99 (0.96-1.02)	0.386
ACEi treatment, yes vs no	0.79 (0.45-1.38)	0.404
ARB treatment, yes vs no	0.47 (0.28-0.80)	0.005
Statin treatment, yes vs no	0.78 (0.46-1.31)	0.342

Results are expressed as hazard ratio (HR) and 95% confidence intervals (95% CI). Abbreviations: CV, cardiovascular; GFR, glomerular filtration rate; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HDL, high-density lipoprotein; LDL, low-density lipoprotein; ACEi, angiotensin-converting enzyme inhibitors; ARB, angiotensin receptor 1 blocker.

RESULTS

The multivariate analysis was adjusted for 10 variables and results of independent interactions are shown in Table 31. The composite outcome non-fatal and fatal CV event was independently associated with advanced age (≥65 years), diabetes and baseline circulating ACE2 activity (≥24.9 RFU/μL/h). Non-CV mortality was also associated with advanced age, and increased creatinine and baseline ACE2 activity. Finally, the composite all-cause mortality and non-fatal CV event, and all-cause mortality were independently associated with advanced age and diabetes, but not with baseline circulating ACE2 activity. For the composite outcome all-cause mortality and non-fatal CV event, smoking habits and creatinine were also independent predictors. Male gender was found to be a predictor of all-cause mortality, while hemoglobin was inversely associated.

Table 31. Multivariate logistic regression to model outcomes at 2 years of follow-up.

		Adjusted analysis	
		HR (95% CI)	p-value
Non-fatal and fatal CV event	**Age, <65 vs ≥65 years**	1.51 (1.03-2.23)	0.036
	Diabetes, yes vs no	2.30 (1.57-3.38)	<0.001
	Baseline circulating ACE2 activity, <24.9 vs ≥24.9 RFU/µL/h	1.62 (1.01-2.60)	0.046
Non-CV mortality	**Age, <65 versus ≥65 years**	4.46 (2.41-8.24)	<0.001
	Creatinine, mg/dL	1.34 (1.10-1.64)	0.003
	Baseline circulating ACE2 activity, RFU/µL/h	1.01 (1.00-1.01)	0.002
All-cause mortality and non-fatal CV event	**Age, <65 versus ≥65 years**	2.30 (1.66-3.20)	<0.001
	Current smoker, yes vs no	1.53 (1.10-2.12)	0.012
	Diabetes, yes vs no	1.91 (1.40-2.61)	<0.001
	Creatinine, mg/dL	1.26 (1.12-1.42)	<0.001
	Baseline circulating ACE2 activity, <24.9 vs ≥24.9 RFU/µL/h	0.26 (0.85-1.79)	0.261
All-cause mortality	**Age, <65 versus ≥65 years**	2.63 (1.68-4.12)	<0.001
	Gender, men vs women	2.20 (1.35-3.59)	0.002
	Diabetes, yes vs no	1.67 (1.09-2.55)	0.018
	Hemoglobin, g/dL	0.73 (0.64-0.84)	<0.001
	Baseline circulating ACE2 activity, <24.9 vs ≥24.9 RFU/µL/h	1.23 (0.74-2.05)	0.429

Results are expressed as hazard ratio (HR) and 95% confidence intervals (95% CI). Abbreviations: CV, cardiovascular.

B.Experimental study

B.I.   Physiological parameters

B.I.a. Animal characteristics

The effect of vitamin D analog administration was tested in a mouse model of type 1 diabetes, the NOD mice. Diabetic mice were treated with paricalcitol (low or high dose), aliskiren alone, or a combination of aliskiren and paricalcitol at high dose. NOR mice were used as non-diabetic controls.

Glucose levels were similar in all diabetic groups at the time of inclusion, and as expected, higher than control mice. At the end of the study, 21 days after diabetes diagnosis, the mean blood glucose levels in all NOD groups were maintained around 30 mM without statistical differences as a consequence of treatments (Table 32).

Table 32. Blood glucose levels at baseline (0 days) and at the end-point of the study (21 days).

	Number	Age (weeks)	Blood glucose (mM) Time = 0 days	Blood glucose (mM) Time = 21 days
CONT	12	21.42±1.01	7.50±0.86	9.30±0.32
C-PARI_H	6	22.02±0.02	7.13±0.71	8.82±0.54
NOD	20	19.78±0.69	25.66±1.66‡	34.92±0.63‡
NOD+PARI_L	21	19.90±0.63	25.41±1.47‡	32.18±1.47‡
NOD+PARI_H	20	20.19±0.58	20.41±2.36‡	32.10±2.16‡
NOD+ALSK	16	21.02±0.59	24.32±2.13‡	32.89±2.12‡
NOD+ALSK+PARI	13	19.19±0.77	25.65±1.46‡	32.59±1.34‡

CONT, control mice; C-PARI_H, control mice given 0.8 μg/kg paricalcitol; NOD+PARI_L, NOD mice given 0.4 μg/kg paricalcitol; NOD+PARI_H, NOD mice given 0.8 μg/kg paricalcitol; NOD+ALSK, NOD mice given aliskiren; NOD+ALSK_PARI_L, NOD mice given 0.4 μg/kg paricalcitol and aliskiren in combination. ‡$P<0.05$ versus CONT.

As shown in Table 33, when kidney weight to body weight ratio was compared between groups, NOD mice exhibited a significant increase compared with NOR mice, indicating an effect of early diabetes on renal hypertrophy. Only treatment with low-dose paricalcitol (NOD+PARI_L) or aliskiren (NOD+ALSK) modulated this effect and significantly decreased the ratio compared with the untreated diabetic group. Heart weight to body weight ratio was not different between control and diabetic groups. However, paricalcitol-treated NOD mice showed significantly reduced heart/body weight ratio compared with the untreated NOD group (Table 33).

ACR in spot urine sample was determined to evaluate albuminuria at the end of the study. In this model of early DN, NOD mice exhibited significantly increased albuminuria as compared with control mice (Table 33). Treatments

RESULTS

(either with paricalcitol or aliskiren) resulted in slightly decreased levels of ACR in diabetic mice compared with untreated diabetic animals, but statistical significance was not reach (Figure 37).

Table 33. Physiological parameters at the end-point of the study.

	Kidney-to-body weight ratio (%)	Heart-to-body weight ratio (%)	UAE (µg albumin/mg creatinine)
CONT	0.90±0.03	0.43±0.02	22.17±7.50
C-PARI_H	0.81±0.06	0.40±0.02	20.96±7.02
NOD	1.61±0.05[‡]	0.40±0.02	482.93±275.41[‡]
NOD+PARI_L	1.51±0.04[‡§]	0.36±0.01[§]	419.68±209.93[‡]
NOD+PARI_H	1.58±0.04[‡]	0.37±0.02[§]	311.93±57.63[‡]
NOD+ALSK	1.51±0.05[‡§]	0.39±0.02	234.41±54.04[‡]
NOD+ALSK+PARI	1.62±0.05[‡]	0.40±0.03	433.10±202.60[‡]

CONT, control mice; C-PARI_H, control mice given 0.8 µg/kg paricalcitol; NOD, diabetic mice; NOD+PARI_L, NOD mice given 0.4 µg/kg paricalcitol; NOD+PARI_H, NOD mice given 0.8 µg/kg paricalcitol; NOD+ALSK, NOD mice given aliskiren; NOD+ALSK_PARI_L, NOD mice given 0.4 µg/kg paricalcitol and aliskiren in combination; UAE, urinary albumin excretion. ‡P<0.05 versus CONT; §P<0.05 versus NOD.
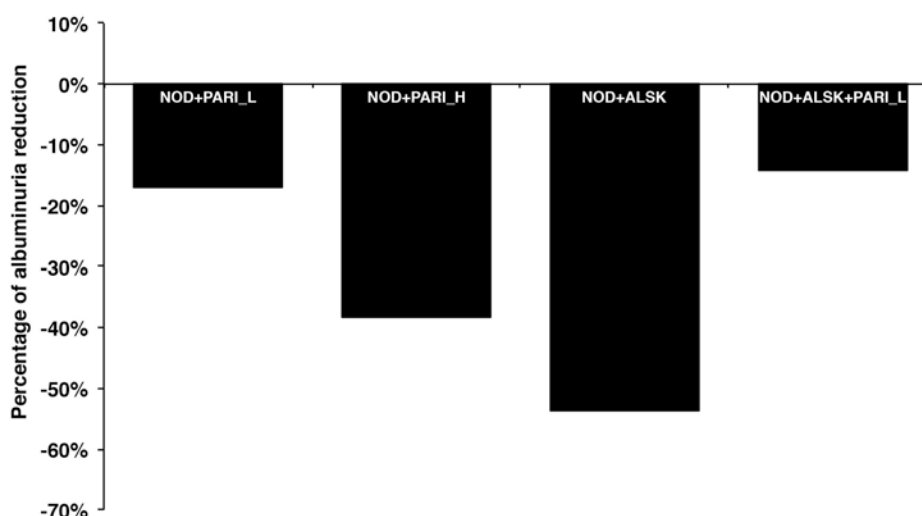
Figure 37. Percentages of reduction in urinary albumin excretion. NOD_PARI_L, NOD mice given 0.4 µg/kg paricalcitol; NOD_PARI_H, NOD mice given 0.8 µg/kg paricalcitol; NOD+ALSK, NOD mice given aliskiren; NOD+ALSK+PARI_L, NOD mice given 0.4 µg/kg paricalcitol and aliskiren.

B.I.b. Blood pressure

SBP and DBP were measured in diabetic and control conscious mice. SBP and DBP were significantly increased in NOD mice compared with control group mice (SBP: 119.13±3.73 mmHg versus 104.62±2.30, P<0.05; DBP: 85.90±3.39 versus 78.59±1.97, P<0.05). However, no effect of paricalcitol was

observed (Figure 38A). As expected, aliskiren treatment resulted in a significant decrease in SBP and DBP compared with untreated diabetic mice (SBP: 98.37±4.61 for NOD+ALSK and 98.12±5.86 for NOD+ALSK+PARI_L; DBP: 73.19±3.42 for NOD+ALSK and 69.96±17.22 for NOD+ALSK+PARI_L). The tail-cuff method also allowed us to measure heart rates in all animals. Heart rate was significantly decreased in NOD mice compared with non-diabetic mice (643.68±13.82 bpm versus 677.42±10.56, P<0.05). No differences were found between untreated and treated diabetic groups (Figure 38B).
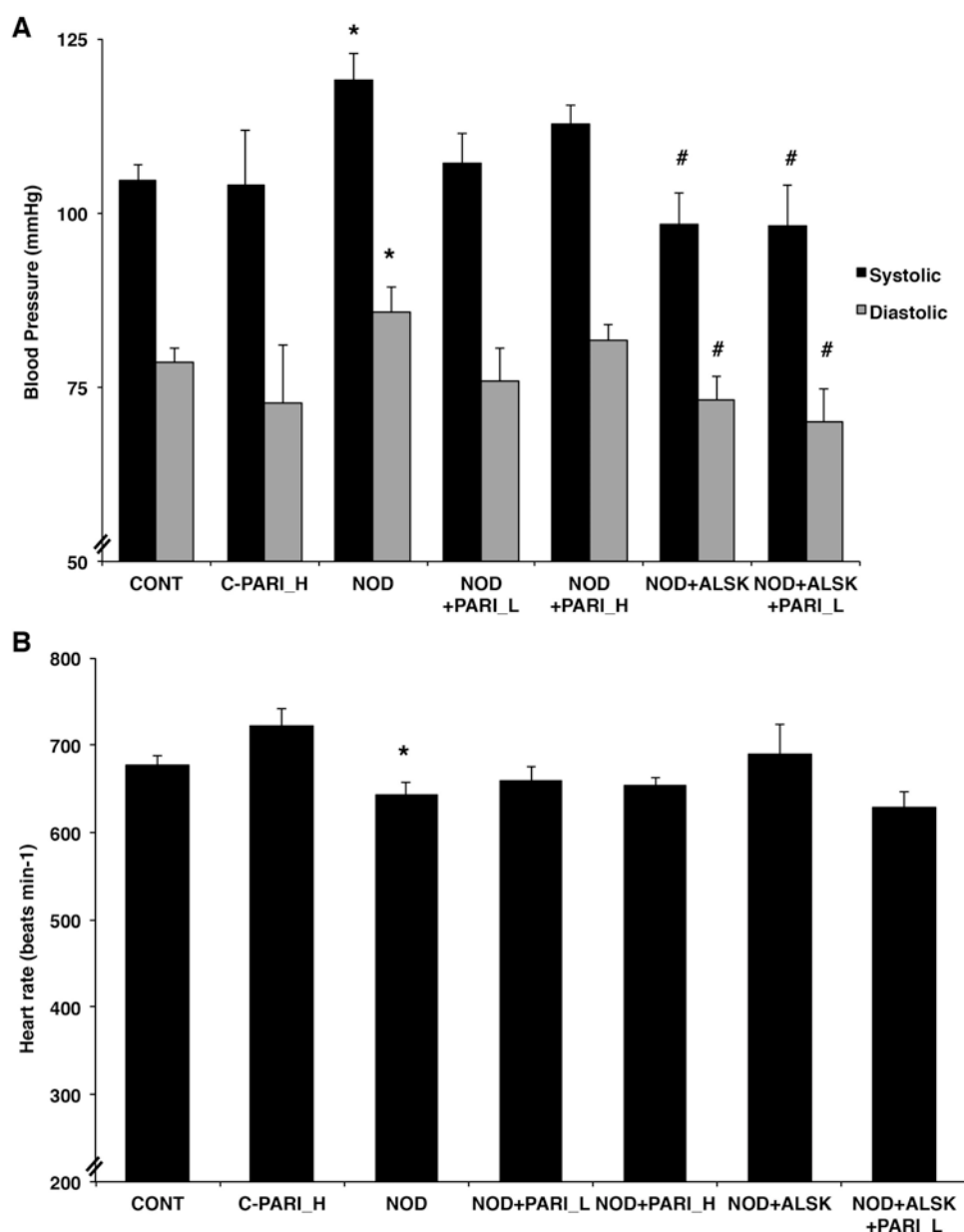
Figure 38. Blood pressure and heart rate parameters. (A) Systolic and diastolic blood pressure values. **(B)** Heart rate values. CONT, control mice; C-PARI_H, control mice given 0.8 μg/kg paricalcitol; NOD, diabetic mice; NOD+PARI_L, NOD mice given 0.4 μg/kg paricalcitol; NOD+PARI_H, NOD mice given 0.8 μg/kg paricalcitol; NOD+ALSK, NOD mice given aliskiren; NOD+ALSK_PARI_L, NOD mice given 0.4 μg/kg paricalcitol and aliskiren. *P<0.05 versus CONT; #P<0.05 versus NOD.

B.II. ACE2 activity and expression

To study the modulation of vitamin D analog treatment on ACE2, enzymatic activity and expression assays were performed in NOD mice (*in vivo*) and in tubular epithelial cells (*in vitro*).

B.II.a. *In vivo* study

Circulating ACE2 was significantly increased in the diabetic group compared with the non-diabetic control group (418.36±42.31 RFU/μL/h versus 112.76±5.52, $p<0.05$) (Figure 39A). Paricalcitol administration resulted in significantly decreased ACE2 activity in all treated mice (NOD+PARI_L: 295.93±17.40; NOD+PARI_H: 301.39±12.38; NOD+ALSK+PARI_L: 263.60±32.54). Interestingly, aliskiren administration alone did not modify ACE2 activity in diabetic mice.

In renal cortex homogenates, ACE2 enzymatic activity was significantly increased in diabetic mice compared with non-diabetic control mice (4542.65±331.85 RFU/μg/h versus 2113.46±166.31, $p<0.05$) (Figure 39B). However, no differences were observed between treated and non-treated diabetic groups.

ACE2 enzymatic activity was also tested in spot urine sample. As shown in serum and renal cortex, ACE2 activity was significantly increased in urine from diabetic mice compared with control mice (6307.89±2005.76 RFU/mgCrea/h versus 123.41±21.24, $p<0.05$) (Figure 39C). No differences were found between treated and non-treated diabetic groups.
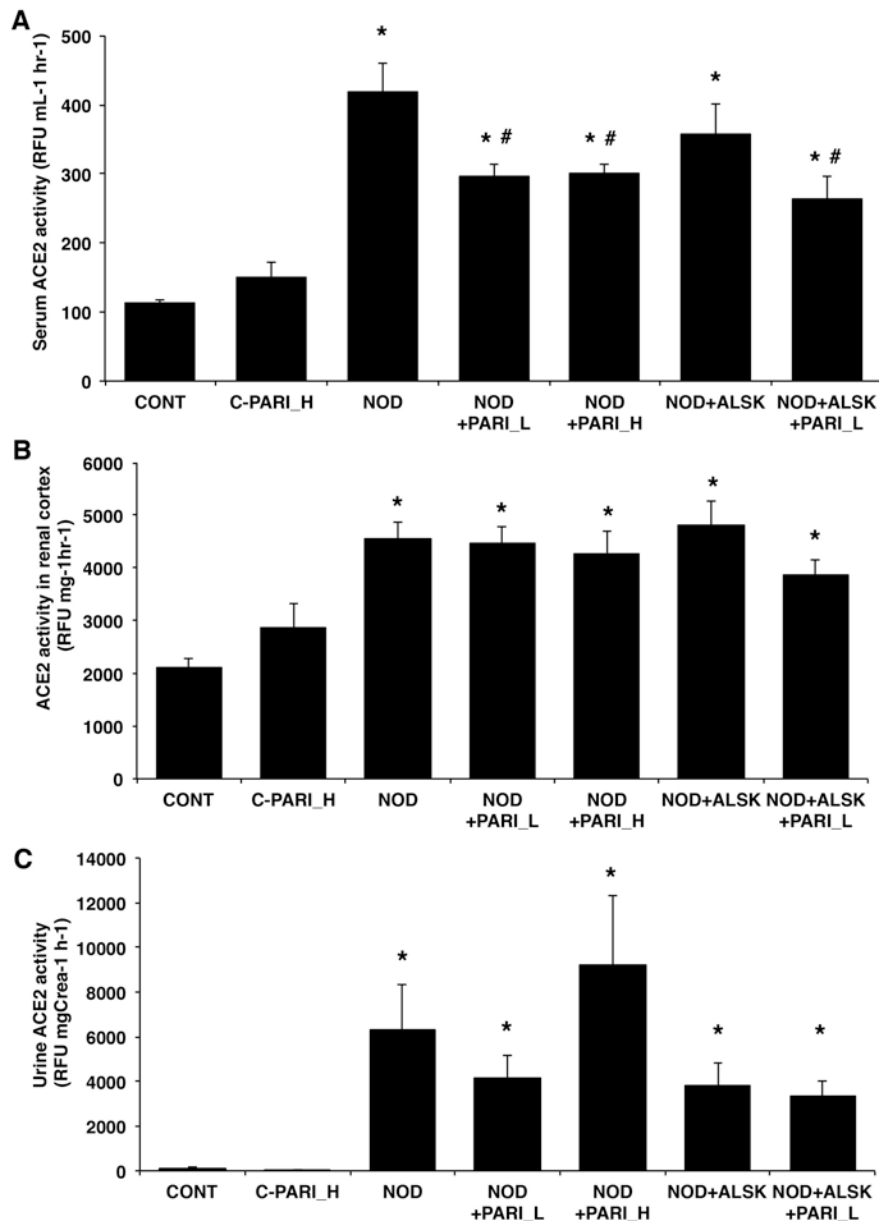
Figure 39. Angiotensin-converting enzyme 2 (ACE2) activity. (A) Serum ACE2 activity; **(B)** ACE2 activity in renal cortex; **(C)** Urine ACE2 activity. RFU, relative fluorescent units. CONT, control mice; C-PARI_H, control mice given 0.8 µg/kg paricalcitol; NOD, diabetic mice; NOD+PARI_L, NOD mice given 0.4 µg/kg paricalcitol; NOD+PARI_H, NOD mice given 0.8 µg/kg paricalcitol; NOD+ALSK, NOD mice given aliskiren; NOD+ALSK_PARI_L, NOD mice given 0.4 µg/kg paricalcitol and aliskiren. *$P < 0.05$ versus CONT; #$P < 0.05$ versus NOD.

ACE2 gene and protein expressions were analyzed in the renal cortex of all animals by real-time PCR (Figure 40A) and Western blot (Figure 40B-C). *Ace2* gene expression levels were similar in diabetic and non-diabetic mice. In all diabetic treated mice *ace2* gene expression was significantly increased as compared with diabetic untreated mice (NOD: 1.09±0.11 versus NOD+PARI_L: 1.62±0.17; NOD+PARI_H: 1.73±0.13; NOD+ALSK: 1.56±0.22; NOD+ALSK+PARI_L: 1.73±0.28, p<0.05) (Figure 40A). The lack of increase in *ace2* gene expression in NOD mice was not translated to the protein level,

where ACE2 protein expression was significantly increased in diabetic mice compared to control mice (0.66±0.07 versus 0.41±0.07, p<0.05) (Figure 40B-C). Paricalcitol administration alone resulted in significantly increased ACE2 protein and gene expressions in kidney cortex in both control and diabetic mice compared with vehicle-treated mice (C-PARI_H: 1.06±0.43; NOD+PARI_L: 0.81±0.06; NOD+PARI_H: 0.87±0.09; NOD+ALSK: 0.70±0.10; NOD+ALSK+PARI_L: 0.77±0.09).
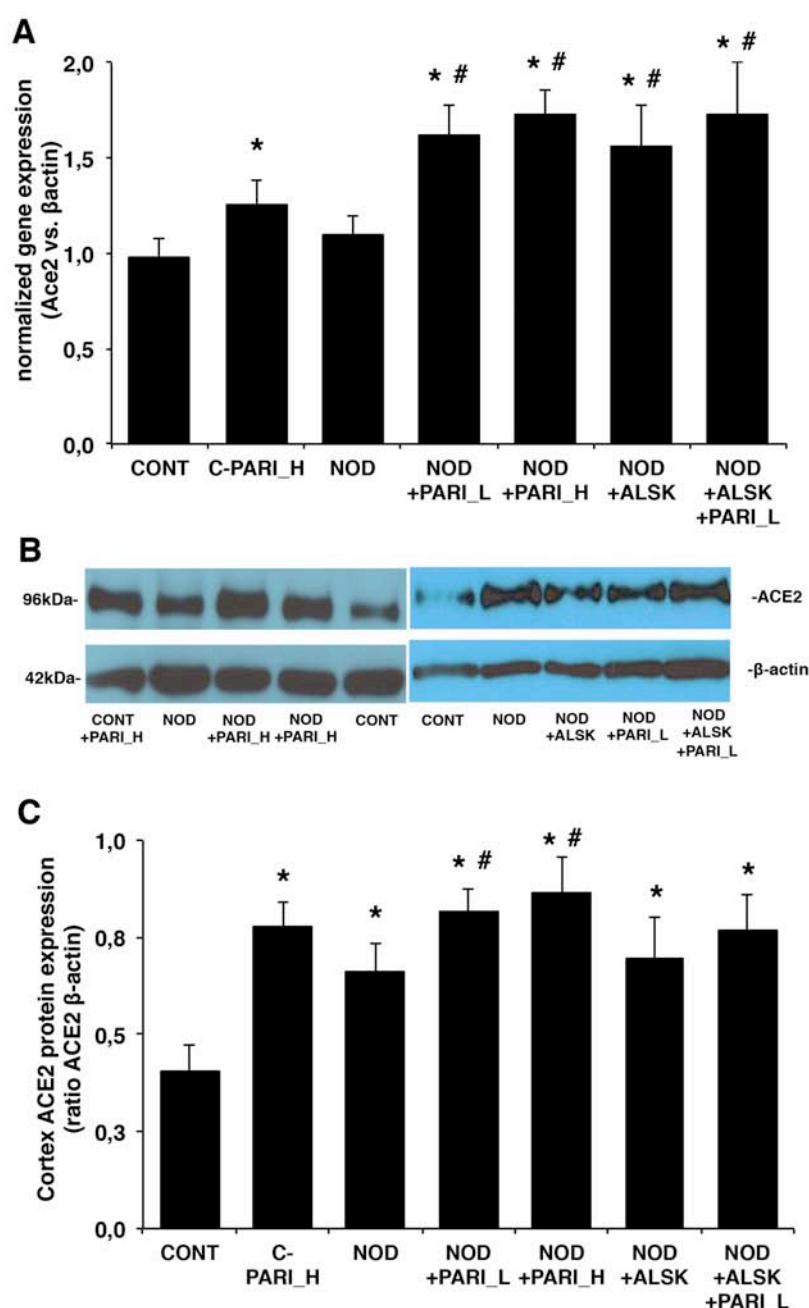
Figure 40. (A) ACE2 gene expression by real time PCR; (B) Representative image of a Western Blog; (C) Protein expression quantification of ACE2 by ImageJ software. CONT, control mice; C-PARI_H, control mice given 0.8 μg/kg paricalcitol; NOD, diabetic mice; NOD+PARI_L, NOD mice given 0.4 μg/kg paricalcitol; NOD+PARI_H, NOD mice given 0.8 μg/kg paricalcitol; NOD+ALSK, NOD mice given aliskiren; NOD+ALSK_PARI_L, NOD mice given 0.4 μg/kg paricalcitol and aliskiren. *P<0.05 versus CONT; #P<0.05 versus NOD.

Immunohistochemistry studies were also performed in renal samples (Figure 41A). Kidney ACE2 staining was observed mainly in the brush border of proximal tubules. The intensity and distribution of the positive signal resembled the profile described by Western blot analysis. ACE2 was significantly increased in diabetic mice compared to control mice (47.73 ± 1.16 AU of MGV versus 42.80 ± 1.71, $p<0.05$) (Figure 41B-C). Paricalcitol administration at high dose significantly increased ACE2 expressions in kidney cortex in diabetic mice compared with vehicle-treated mice (NOD+PARI_H: 55.79 ± 1.30).
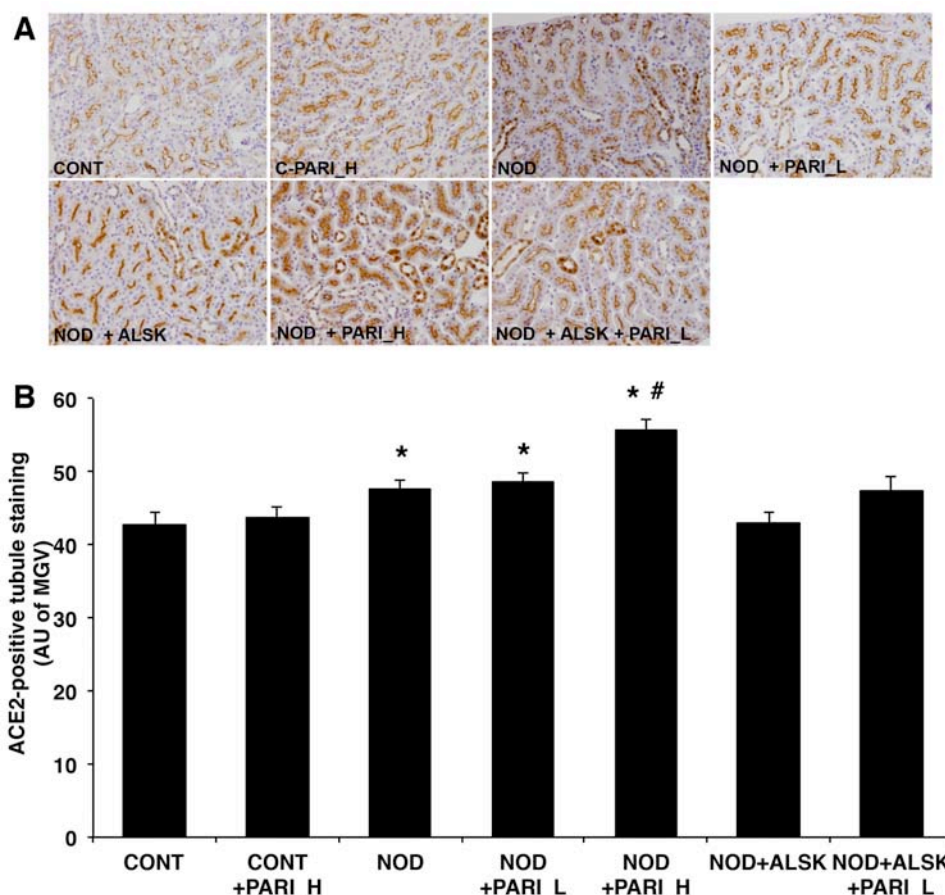
Figure 41. Immunohistochemistry analysis of ACE2 protein expression in renal cortex. AU of MGV, arbitrary units of mean gray value; CONT, control mice; C-PARI_H, control mice given 0.8 μg/kg paricalcitol; NOD, diabetic mice; NOD+PARI_L, NOD mice given 0.4 μg/kg paricalcitol; NOD+PARI_H, NOD mice given 0.8 μg/kg paricalcitol; NOD+ALSK, NOD mice given aliskiren; NOD+ALSK_PARI_L, NOD mice given 0.4 μg/kg paricalcitol and aliskiren. *P<0.05 versus CONT; #P<0.05 versus NOD.

B.II.b. *In vitro* study

To test the direct effect of paricalcitol on renal tubular epithelial cells, cultured MTC cells in high-glucose medium were exposed to increasing doses of paricalcitol for 24 hours. Paricalcitol at 0.4 (29371 ± 6260.44 arbitrary units of fluorescence per cell) and 4.0 ng/mL (38170.83 ± 8402.98) resulted in significantly increased ACE2 expression in renal tubular epithelial cells

compared with control cells (13998.58±2344.80, p<0.05) (Figure 42). Thus, incubation with paricalcitol resulted in increased ACE2 expression in a dose-dependent manner.
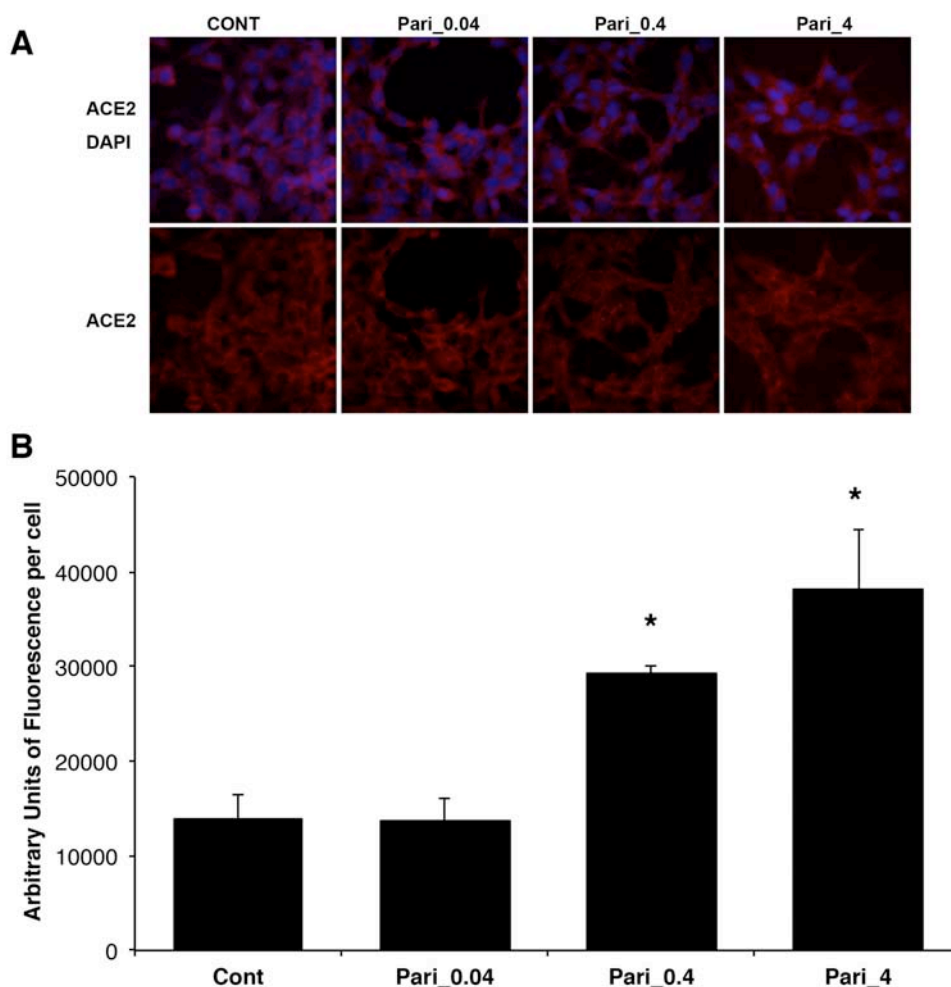
Figure 42. ACE2 protein expression in MTC cells. Cont, MTC cells incubated in high-glucose medium; Pari_0.04, MTC cells incubated in high-glucose medium and 0.04 ng/mL of paricalcitol; Pari_0.4, MTC cells incubated in high-glucose medium and 0.4 ng/mL of paricalcitol; Pari_4, MTC cells incubated in high-glucose medium and 4.0 ng/mL of paricalcitol.

B.III. ACE activity and expression

To elucidate the possible interplay of ACE2 with other RAS components we examined the modification in ACE, not only in serum and cortical enzyme activity, but also in gene and protein expression. As shown in Figure 43A, serum ACE activity was significantly increased in diabetic mice as compared with control mice (2191.89±125.52 RFU/μL/min versus 1483.66±106.14, p<0.05). No differences were observed in treated diabetic groups.

ACE activity in renal cortex was significantly decreased in diabetic mice as compared with control mice (1789.24±111.96 versus 2345.16±171.12,

p<0.05) (Figure 43B). Administration of high-dose paricalcitol resulted in increased ACE activity to levels of control mice (NOD+PARI_H: 2219.95±121.52).

Regarding ACE protein and gene expression, no differences were found between diabetic and control groups (Figure 43C-E). Administration of high-dose paricalcitol resulted in significantly higher ACE gene expression in renal tissue as compared to the untreated diabetic mice (4.92±0.33 versus 2.82±0.26, p<0.05) (Figure 43E).
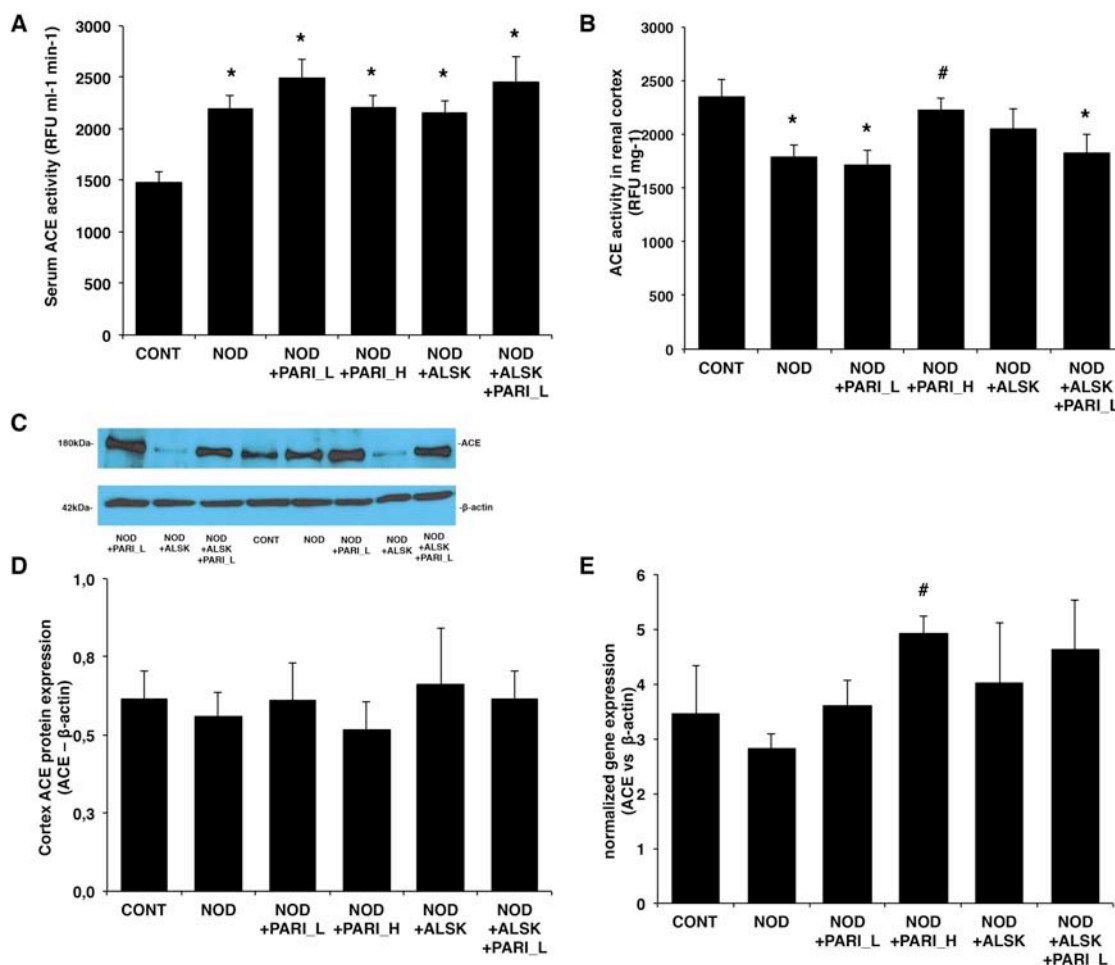
Figure 43. ACE expression and activity. (A) Serum ACE activity; **(B)** ACE activity in renal cortex; **(C)** Representative image of a Western Blot; **(D)** Protein expression quantification by ImageJ software; **(E)** Gene expression of ACE from renal cortex. CONT, control mice; NOD, diabetic mice; NOD+PARI_L, NOD mice given 0.4 μg/kg paricalcitol; NOD+PARI_H, NOD mice given 0.8 μg/kg paricalcitol; NOD+ALSK, NOD mice given aliskiren; NOD+ALSK_PARI_L, NOD mice given 0.4 μg/kg paricalcitol and aliskiren. *P<0.05 versus CONT; #P<0.05 versus NOD.

B.IV. Renin activity and expression

Given that previous studies have shown the effect of paricalcitol in reducing cortical renin gene expression, this study also explored this effect. Renin activity was first tested by using recombinant renin and aliskiren. In addition, the direct effect of aliskiren on reducing renin activity was also assessed in the NOD group (Figure 44).
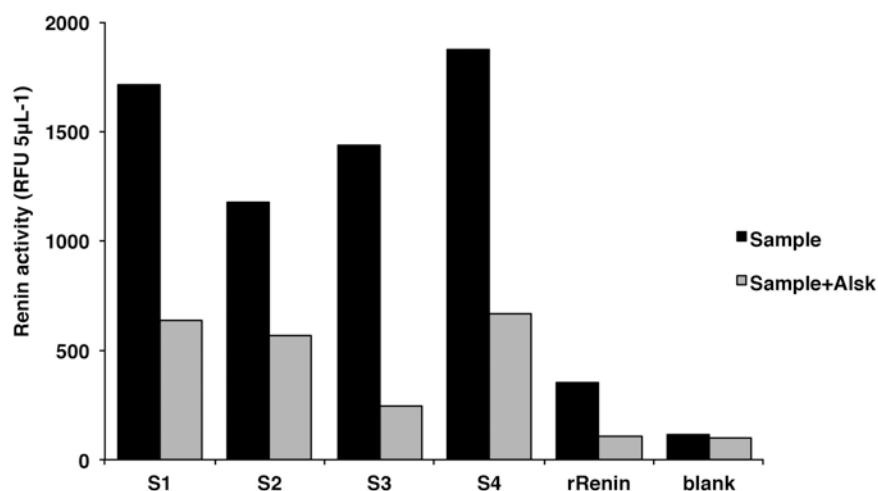
Figure 44. Validation assay of renin activity by incubation of samples from the NOD group (S1-S4) and recombinant renin (rRenin) with aliskiren. Addition of aliskiren to the NOD samples and to the recombinant renin (rRenin) resulted in reduced renin activity.

Regarding serum renin activity, untreated diabetic mice exhibited a significant increase compared with controls (NOD: 1941±122; NOR: 1409±72.09). Administration of aliskiren (alone or in combination with paricalcitol) resulted in a significant decrease in renin activity in the treated diabetic mice (NOD+ALSK: 1623±130; NOD+ALSK+PARI_L: 1507±111) (Figure 45A). Gene expression analysis showed no differences between diabetic and control mice (NOD: 0.47±0.04; NOR: 0.79±0.21) (Figure 45B). In concordance with previous studies, administration of high-dose paricalcitol resulted in significantly decreased renin gene expression in diabetic mice (NOD+PARI_H: 0.34±0.03). In contrast, mice receiving aliskiren exhibited a large increase in renin gene expression compared with untreated diabetic and control mice (NOD+ALSK: 2.09±0.38; NOD+ALSK+PARI_L: 1.55±0.35).
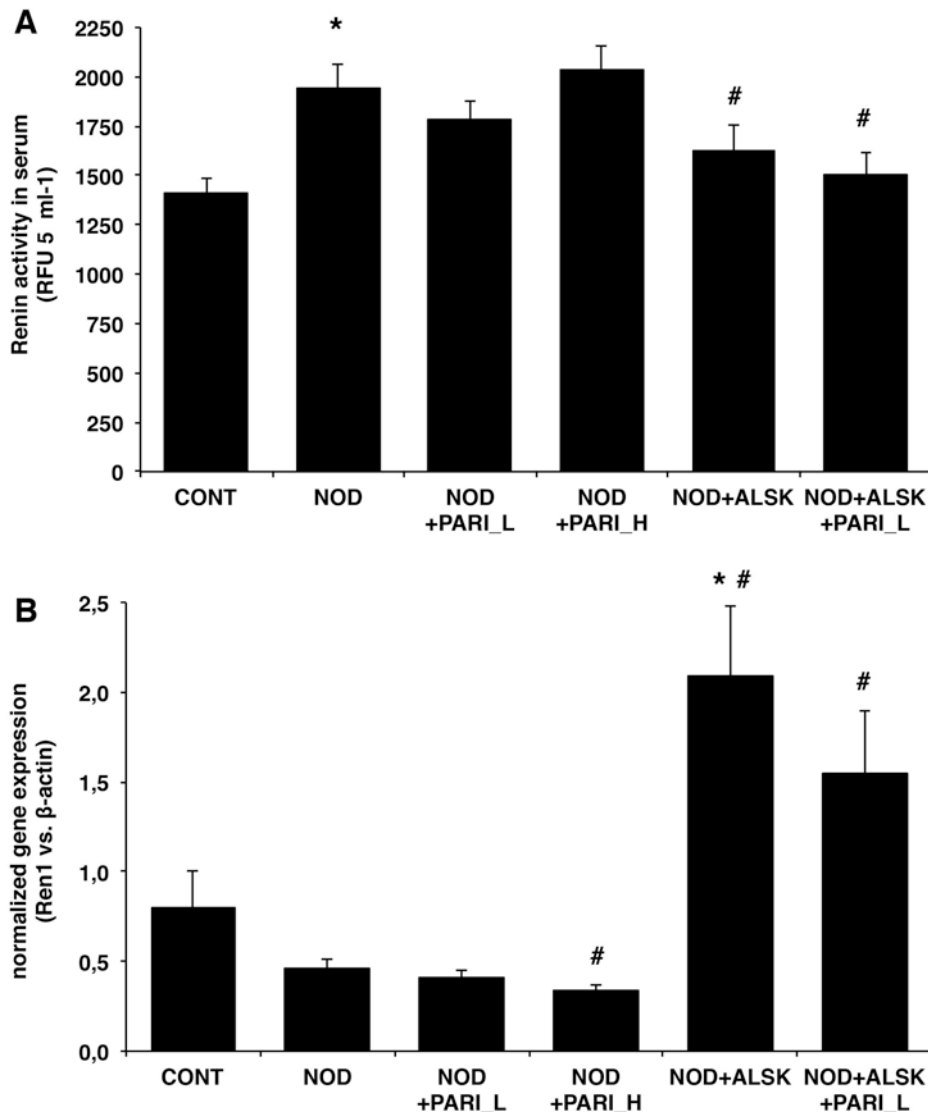
Figure 45. Renin activity and expression (A) Serum renin activity. **(B)** Renin gene expression. CONT, control mice; NOD, diabetic mice; NOD+PARI_L, NOD mice given 0.4 µg/kg paricalcitol; NOD+PARI_H, NOD mice given 0.8 µg/kg paricalcitol; NOD+ALSK, NOD mice given aliskiren; NOD+ALSK_PARI_L, NOD mice given 0.4 µg/kg paricalcitol and aliskiren. *P<0.05 versus CONT; #P<0.05 versus NOD.

Renin immunohistochemistry revealed a significant increase in protein expression in the renal cortex (both in tubule and juxtaglomerular apparatus) in aliskiren-treated mice compared with NOD mice (NOD+ALSK: 31.82±1.32 AU of MGV; NOD+ALSK_PARI_L: 21.29±3.02; NOD: 9.32±1.16 in tubule / NOD+ALSK: 886.04±39.13; NOD+ALSK_PARI_L: 594.38±46.99; NOD: 463.17±18.48 in juxtaglomerular apparatus) (Figure 46A-D). In contrast, high-dose paricalcitol induced different patterns of renin expression in the tubule than in the juxtaglomerular apparatus: although it led to higher expression in the tubule, no changes were observed in the juxtaglomerular apparatus (NOD+PARI_L: 9.25±1.73; NOD+PARI_H: 14.66±2.30 in tubule / NOD+PARI_L: 409.72±18.29; NOD+PARI_H: 500.43±25.62 in juxtaglomerular apparatus) (Figure 46A-D).
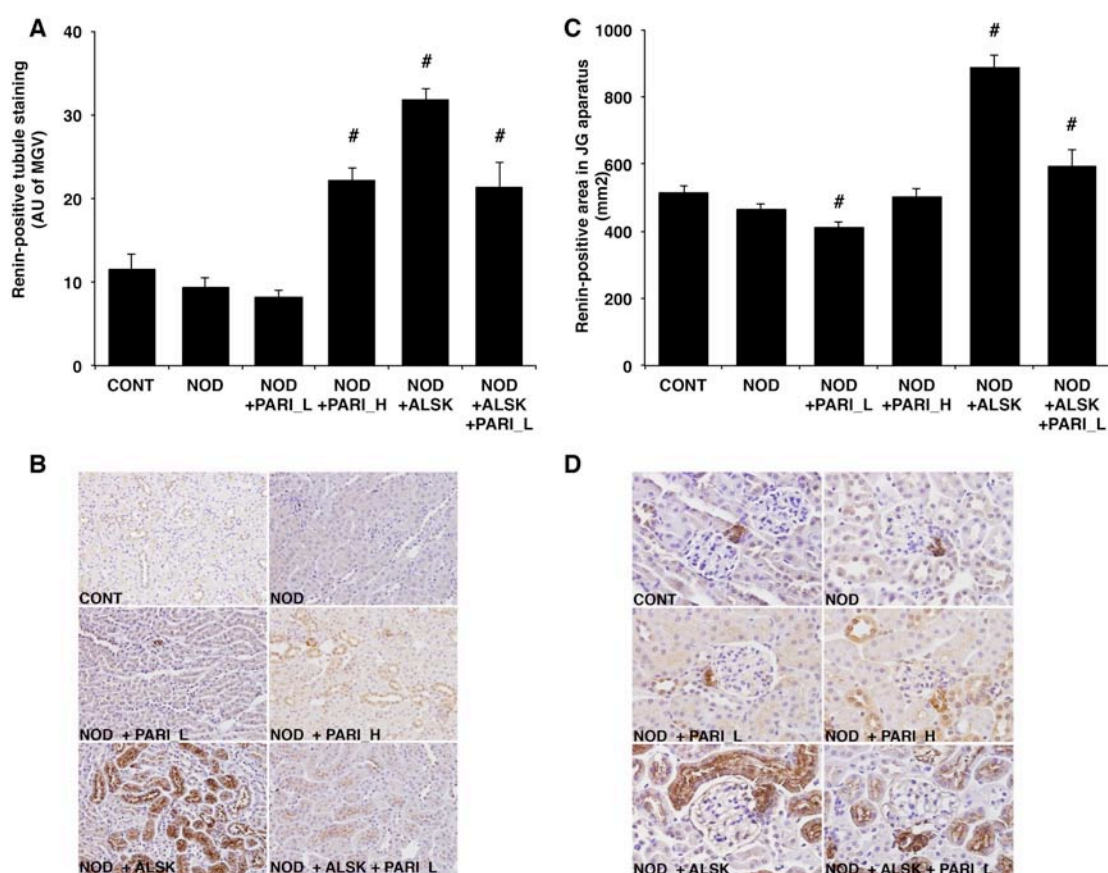
Figure 46. Renal renin expression in **(A-B)** cortical tubules and **(C-D)** juxtaglomerular apparatus. AU of MGV, arbitrary units of mean gray value; CONT, control mice; NOD, diabetic mice; NOD+PARI_L, NOD mice given 0.4 μg/kg paricalcitol; NOD+PARI_H, NOD mice given 0.8 μg/kg paricalcitol; NOD+ALSK, NOD mice given aliskiren; NOD+ALSK_PARI_L, NOD mice given 0.4 μg/kg paricalcitol and aliskiren. *$P<0.05$ versus CONT; #$P<0.05$ versus NOD.

B.V. Renal Ang II and Ang 1-7 levels

Renal levels of Ang II and Ang 1-7 were measured in renal cortex from all diabetic mice (Figure 47). Ang II levels were significantly decreased in the group receiving paricalcitol and aliskiren in combination as compared with untreated diabetic mice (NOD+ALSK+PARI_L: 0.08±0.02; NOD: 0.24±0.03) (Figure 47A). No differences were found in the other treated groups. Regarding Ang 1-7 renal content, the aliskiren-treated group exhibited a significantly lower level as compared with untreated diabetic mice (NOD+ALSK: 0.76±0.06; NOD: 1.27±0.13) (Figure 47B). The Ang 1-7 and Ang II ratio was significantly greater in the group that received paricalcitol and aliskiren in combination as compared to the NOD group (NOD+ALSK+PARI_L: 38.12±16.10; NOD: 6.04±1.05) (Figure 47C).
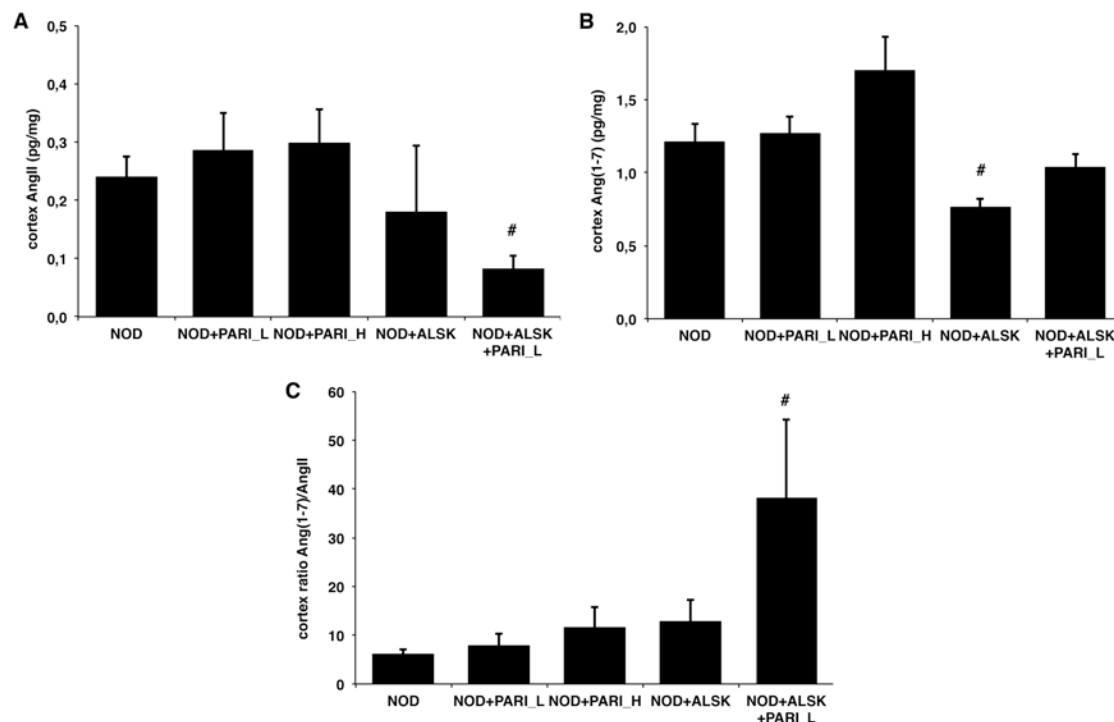
Figure 47. Renal Ang II and Ang 1-7 content. (A) Ang II levels in renal cortex; **(B)** Ang 1-7 levels in renal cortex; **(C)** ratio of Ang 1-7 and Ang II. NOD, diabetic mice; NOD+PARI_L, NOD mice given 0.4 µg/kg paricalcitol; NOD+PARI_H, NOD mice given 0.8 µg/kg paricalcitol; NOD+ALSK, NOD mice given aliskiren; NOD+ALSK_PARI_L, NOD mice given 0.4 µg/kg paricalcitol and aliskiren. [#]$P<0.05$ versus NOD.

B.VI. Oxidative stress

Oxidative stress has also been implicated in the pathogenesis of renal injury mediated by RAS peptides. Thus, two different analysis of oxidative stress were performed: measurement of H_2O_2 in serum and nitrotyrosine in paraffin-embedded kidney tissue from diabetic mice. Regarding H_2O_2, administration of high-dose paricalcitol or paricalcitol in combination with aliskiren resulted in significantly decreased levels of circulating H_2O_2 compared with untreated diabetic mice (NOD+PARI_H: 14.16±1.60 mM; NOD+ALSK+PARI_L: 13.82±1.07; NOD: 16.43±2.08) (Figure 48A). Similarly, administration of high-dose paricalcitol also resulted in significantly decreased levels of nitrotyrosine staining compared with untreated diabetic mice (NOD+PARI_H: 4177.46±591.42 AU of MGV; NOD: 10029.78±1628.67) (Figure 48B-C).
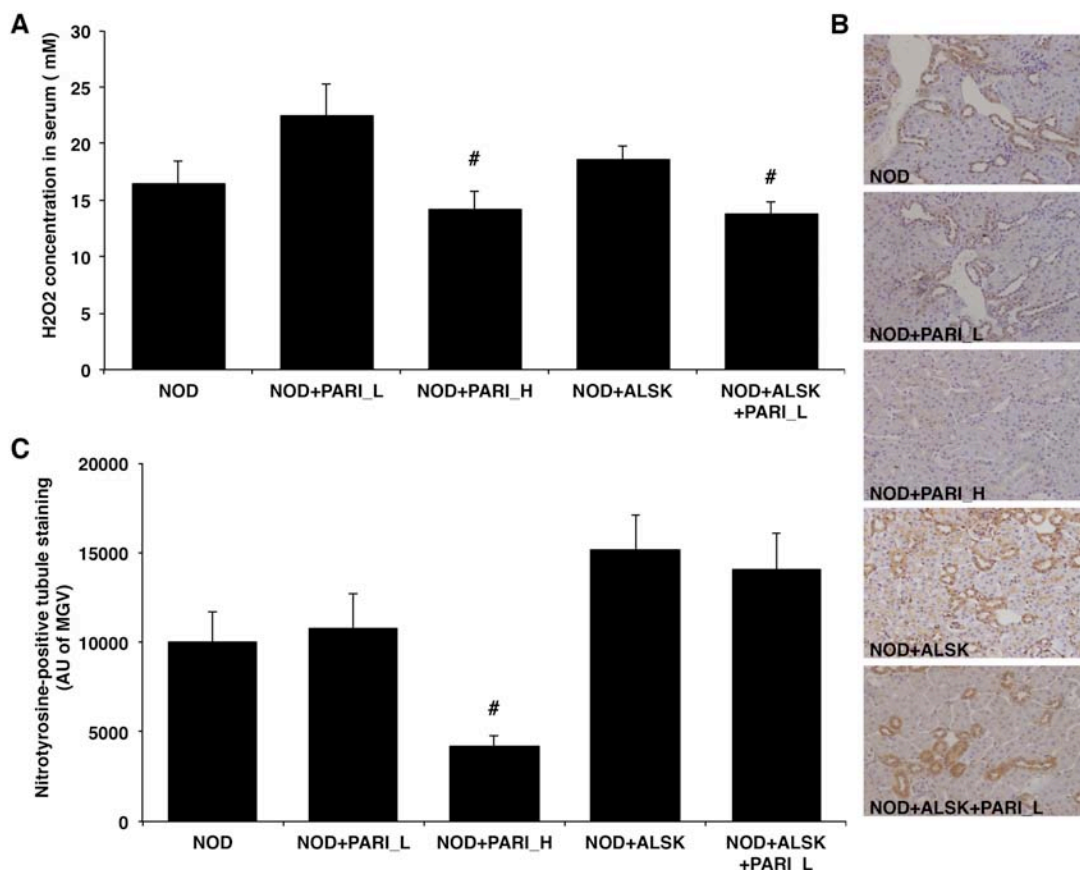
Figure 48. Oxidative stress determination. (A) Hydrogen peroxide (H_2O_2) concentration in serum from NOD mice. **(B and C)** Quantification of Nitrotyrosine in renal cortex from NOD mice. AU of MGV, arbitrary units of mean gray value; NOD, diabetic mice; NOD+PARI_L, NOD mice given 0.4 μg/kg paricalcitol; NOD+PARI_H, NOD mice given 0.8 μg/kg paricalcitol; NOD+ALSK, NOD mice given aliskiren; NOD+ALSK_PARI_L, NOD mice given 0.4 μg/kg paricalcitol and aliskiren. [#]$P<0.05$ versus NOD.

B.VII. Renal ADAM17 content

It has been previously reported that ADAM17 is one of the metalloproteinases implicated in ACE2 shedding. For this reason, we determined ADAM17 content in renal cortex extracts from each group (Figure 49). As expected, untreated diabetic mice exhibited higher levels of ADAM17 than non-diabetic control mice (NOD: 75.01±7.00 pg/μg protein; NOR: 37.56±3.53). Administration of high-dose paricalcitol showed a significant decrease in renal ADAM17 content to levels of non-diabetic control mice compared with those in untreated diabetic mice (NOD+PARI_H: 40.57±4.42). Other treatments resulted in only slightly decreased levels of ADAM17 in renal cortex of diabetic mice, without statistical differences.
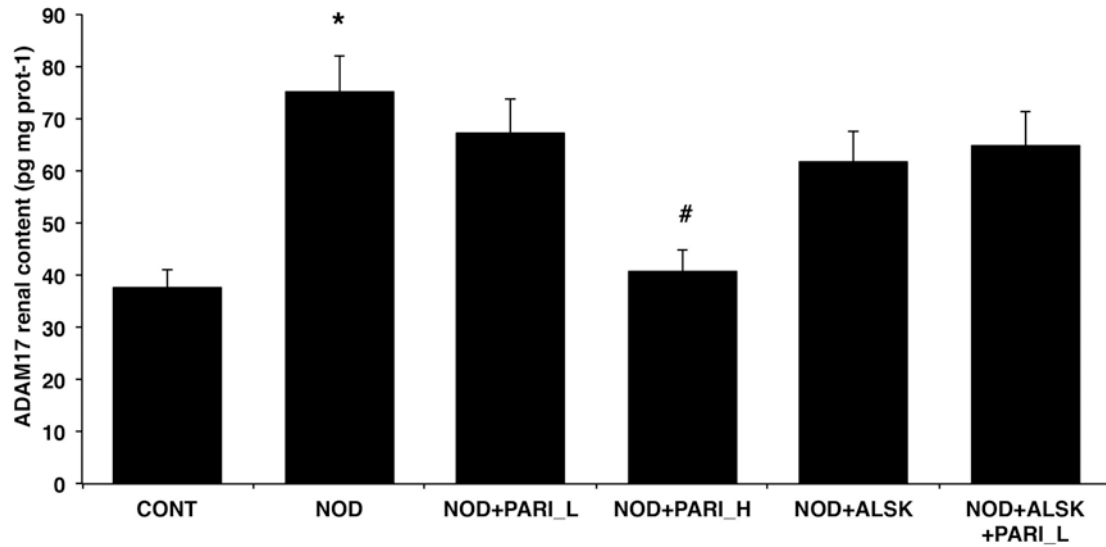
Figure 49. ADAM17 renal content determined by ELISA. CONT, control mice; NOD, diabetic mice; NOD+PARI_L, NOD mice given 0.4 μg/kg paricalcitol; NOD+PARI_H, NOD mice given 0.8 μg/kg paricalcitol; NOD+ALSK, NOD mice given aliskiren; NOD+ALSK_PARI_L, NOD mice given 0.4 μg/kg paricalcitol and aliskiren. *P<0.05 versus CONT; #P<0.05 versus NOD.

VI. DISCUSSION

VI. DISCUSSION

A. Human study

ACE2 enzymatic activity has been widely studied in renal, heart, and other tissues under physiological and pathological conditions [88,166,211]. Several studies have also analyzed human circulating ACE2 activity. However, the majority of them have measured ACE2 activity in serum [159,160,167] or heparin blood samples [155,156]. In this work, blood samples from patients included in the NEFRONA Study were collected in tubes containing EDTA. It has been previously reported that the metal-chelating agent, EDTA completely inhibits ACE2 activity from CHO cells medium and from renal tissue [118,166]. In addition, EDTA has been used to validate ACE2 activity assays [118,166]. Therefore, initially we were not able to detect ACE2 activity in EDTA-plasma samples from study subjects. However, we have been the first to demonstrate that addition of zinc chloride competes with EDTA from plasma samples to bind to the catalytic site (Zn-binding site) of ACE2 and ACE, blocking the Zn-binding site from EDTA and allowing us to measure ACE2 and ACE activities in human EDTA-plasma samples. This finding is of great interest because it allows retrospective analysis of circulating ACE2 activity in stored EDTA-plasma samples.

Few studies have assessed the role of ACE2 activity in CKD patients. To date, there is only one study examining whether the levels of circulating ACE2 differed between pre-dialysis patients and patients undergoing maintenance hemodialysis [168]. Baseline results from our work initially showed that circulating ACE2 activity is decreased in dialysis patients as compared to CKD. Measurement of circulating ACE2, as well as ACE, pre- and post-dialysis showed no differences, demonstrating that the enzyme is not removed by dialysis. High-molecular weight molecules are thought to be difficult to remove by any dialysis strategy [212]. Thus, we surmise that hemodialysis itself could not alter the levels of the ACE2 or ACE activity in plasma owing to their large molecular size (92.46 and 149.7 kDa, respectively) [213].

In our study, subjects included were CKD patients without history of previous CV disease. CKD5D patients were younger, thinner and had less prevalence of diabetes, hypertension and dyslipidemia than CKD3-5 patients. This could be explained by an incidence-prevalence bias, since as renal dysfunction progresses, patients with worse CV health have a lower possibility of staying free of CV events, and hence, of being recruited for the NEFRONA Study. Given that the majority of these factors have been previously associated with increased levels of circulating ACE2 activity [155,159,167], paired case-control studies were performed. When patients were equally distributed by gender, diabetes, hypertension, dyslipidemia, smoking habits, weight and age, the differences among the CKD groups (dialysis and CKD3-5)

were not observed. Thus, these results suggested that the decrease in circulating ACE2 activity within dialysis patients might be ascribed to patients' characteristics and not to a direct modulation of plasma ACE2 activity. Furthermore, within the NEFRONA population, a significant difference in the levels of circulating ACE2 activity was demonstrated between males and females, with higher levels in males. These results confirm that circulating ACE2 activity is increased in male patients with diabetes [167], CKD [168] and kidney transplant [160].

The RAS is a major hormonal system involved in the pathophysiology of CV disease. Within the RAS, ACE2 counterbalances the vasoconstrictor adverse effects of Ang II by converting it into Ang 1-7. Thus, circulating ACE2 has been proposed as an emergent biomarker for CV disease [214]. An initial study in a large cohort from healthy subjects and their first-, second-, or third-degree relatives (537 subjects) was able to detect circulating ACE2 activity in only 40 subjects, older and with a higher prevalence of CV disease, diabetes, and hypertension than those with undetectable ACE2 [155]. Subsequent studies demonstrated that circulating ACE2 activity could be detected both in patients with CV disease and in healthy subjects [156]. The study by Epelman et al. confirmed that circulating ACE2 activity could be measured in heterogeneous patient populations and that it was elevated in human heart failure [157]. In addition, ACE2 correlated independently with worsening disease severity as defined by left ventricular ejection fraction and New York Heart Association functional class. Further studies showed that plasma ACE2 activity increased in patients with left ventricular systolic dysfunction, an important risk factor for coronary heart disease, sudden death, heart failure and stroke [158]. Increased circulating ACE2 activity was also found to be an independent predictor of the combined endpoint of death, cardiac transplant and heart failure hospitalizations, suggesting the potential importance of ACE2 in the development and progression of heart failure [158]. In concordance, a more recent study in patients with ST-elevation myocardial infarction (STEMI) showed that ACE2 was upregulated in the acute phase of STEMI and that it correlated with the infarct size [159]. Soro-Paavonen et al. demonstrated that ACE2 activity was increased in patients with diabetes, vascular complications and decreased estimated GFR [167]. In agreement, our results show that patients with atherosclerotic plaques (CONT, CKD3-5 and CKD5D) had increased levels of circulating ACE2 activity as compared to patients without plaques. Furthermore, in multivariate analysis circulating ACE2 activity was independently associated with the classical CV risk factors: male gender, advanced age and diabetes in CKD3-5, and male gender and advanced age in CKD5D patients. Importantly, these associations of circulating ACE2 activity with classical CV risk factors are the first reported in CKD patients without previous history of CV disease.

Regarding circulating ACE activity, some studies have reported lower levels of ACE in subjects with history of hypertension [155]. In patients with myocardial infarction, plasma ACE levels decreased with age [215]. Plasma

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ACE levels are higher in subjects with nephropathy than in those without it [216]. However, in other studies, no relationship between circulating ACE and the classical CV risk factors was found [160,217]. We have shown that circulating ACE activity is increased in CKD3-5 and CKD5D patients without previous history of CV disease and that it negatively correlates with the classical CV risk factors, such as male gender and older age in CKD3-5. As expected, patients in treatment with ACE inhibitors showed decreased ACE activity in all groups. Thus, the disagreement observed among studies and populations may be ascribed to the effect of RAS blockade on circulating ACE. In the NEFRONA Study, RAS blockade agents were not discontinued during the study, which might have an influence on the negative association of circulating ACE with CV risk factors.

CV disease is the leading cause of morbidity and mortality in CKD patients [30–32]. However, although clinical CV outcomes, such as heart attack, stroke and sudden cardiac death, have a dramatic onset, they result from prolonged exposure to risk factors. In this regard, noninvasive procedures, such as ABI or IMT measurements, to detect silent atherosclerosis have been proposed to estimate the CV risk [218]. ABI is a clinical tool for the detection of peripheral arterial disease, which in turn predicts CV morbidity and mortality. Both low (<0.9) and high (>1.4) ABI values have been associated with high CV mortality and patients in high-risk groups with a low ABI tend to be older, hypertensive, diabetic and have both dyslipidemia and microalbuminuria [219]. Carotid IMT measured using ultrasonography assesses the extent and severity of atherosclerosis. It has been described as an independent predictor of CV death in dialysis patients [220]. In addition, the study by Kato et al. showed that the only traditional risk factors associated with increased IMT were age and total cholesterol level [220]. However, other studies in CKD patients have found no correlation between increased carotid IMT and traditional risk factors, such as smoking or diabetes [221].

Studies in the NEFRONA population have been focused on studying the prevalence of silent atherosclerosis in CKD patients as compared to healthy population. In this population without previous history of CV disease, the absolute and adjusted prevalence rates of atheromatous plaques were higher among CKD patients than in the non-CKD population [42]. Furthermore, the prevalence was higher as the degree of CKD was more severe, with the highest prevalence among dialysis patients [42]. In this study, diabetes was associated with a higher prevalence of atheromatous plaques among both sexes and at any stage of CKD, although the well known protective effect of female sex was present in CKD patients [42]. In this study, no difference was found in carotid IMT between CKD patients and controls, unless plaque presence was corrected to a higher carotid IMT value [43]. Interestingly, there was a high rate of femoral plaque, even in patients with no carotid atheromatosis. In the NEFRONA Study, CKD was also associated with a pathologic ABI, with a different frequency depending on the range: advances stages of CKD were related with a pathologic ABI in the higher rank (>1.4)

than in the ischemic range (<0.9) [43]. As expected, the prevalence of carotid AD was more than 2-fold higher in patients with DN as compared to those with any causes of kidney disease, suggesting that patients with DN at any grade of CKD are at particularly high risk for subclinical AD [222]. Moreover, in the context of diabetes, the prevalence of silent AD increased with the severity of CKD, confirming our previous findings in the whole cohort [222]. Recently, atheromatosis progression has been assessed in the NEFRONA population during 24 months of follow-up. The main findings were: (1) a high prevalence of CKD patients with atheromatous plaque in carotid and/or femoral territories: (2) progression of plaque across CKD stages associated with the presence of diabetes; (3) risk factors predicting atheromatosis progression different depending on the CKD stage; and (4) progression of atheromatosis associated with progression of CKD [44]. In concordance with these results, we have found that in a subpopulation from the NEFRONA Study (CKD3-5 patients), the number of territories with plaques at 24 months of follow-up was associated with male gender, older age, diabetes, hypertension, dyslipidemia and smoking. Furthermore, baseline circulating ACE2 activity was also increased in CKD3-5 patients with higher number of territories with plaques, as well as in those patients with an increase in the number of territories with plaques over the 2-year follow-up study. Other markers of silent atherosclerosis have also been associated with higher baseline circulating ACE2 activity. We have found a correlation between baseline ACE2 activity and IMT at 24 months of follow-up. In addition, patients with a pathological ABI or with severe AD at 24 months showed higher levels of baseline circulating ACE2.

Presence of ACE2 has been confirmed in endothelial cells and smooth muscle cells from intra-myocardial vessels and cardiac myocytes [148]. In addition, it was found in atherosclerotic lesions from thoracic aorta in rabbits and in human atherosclerotic plaques [149,150]. Crackower et al. confirmed the association of ACE2 with cardiac function in ACE2 KO mice, where the loss of ACE2 resulted in profound contractile dysfunction [119]. In a rabbit atherosclerosis model, local overexpression of ACE2 significantly inhibited the development of early atherosclerotic lesions. The antiatherosclerotic effect of ACE2 was associated with prohibited proliferation and migration of vascular smooth muscle cells and improved endothelial functions [223]. Aortas from ACE2 KO mice exhibited impaired endothelium-dependent vasodilation and decreased tube formation and migration. On the contrary, overexpression of ACE2 improved endothelial cell migration and tube formation [224]. In addition, endothelial Ang II-induced ROS generation was attenuated by ACE2, an action that was attenuated when an antagonist of the Ang 1-7 receptor was administered, suggesting that ACE2 functions largely through an Ang 1-7 dependent pathway [224]. In addition, inhibition of Ang II-induced inflammation by ACE2 overexpression inhibited early atherosclerotic lesions and enhanced stability of atherosclerotic plaques [225,226]. In our study, we have observed that increased number of territories with plaques at 24 months of follow-up is independently associated with elevated baseline circulating

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ACE2 activity in CKD3-5 patients, as well as with male gender, older age and diabetes. The association found between baseline circulating ACE2 activity and the number of territories with plaques at 24 months has allowed us to determine a cut-off value of 24.9 RFU/μL/h for ACE2 activity in this population. This result suggests that CKD3-5 patients with a baseline ACE2 activity higher than 24.9 RFU/μL/h might be at higher risk of atheromatosis progression. Therefore, the well-known association of circulating ACE2 activity with CV disease and the role of silent atherosclerosis in the development of CV disease in CKD patients together with our results suggest that baseline ACE2 activity might serve as a biomarker of 2-year atheromatosis progression.

In patients with chronic systolic heart failure, increased levels of circulating ACE2 activity were associated with all-cause of mortality [158]. In our study, we have analyzed whether circulating ACE2 activity at baseline may serve as a potential predictor of CV events and mortality over 2 and 4 years follow-up in CKD3-5 patients. Baseline ACE2 activity was significantly associated with CV outcomes at 4 years. However, no differences were found between baseline ACE2 activity and CV outcomes at 2 years of follow-up. Interestingly, univariate analysis showed that increased baseline ACE2 activity was associated with non-CV and all-cause mortality both at 2 years and 4 years of follow-up. The lack of association between baseline ACE2 and CV outcomes at 2 years of follow-up may be ascribed to the short-term follow-up and to the low number of CV events observed, which may be influenced by the fact that patients included in the NEFRONA Study had no previous history of CV disease. As expected, during the 4 years of follow-up more CV outcomes occurred, resulting in a significant difference in the levels of baseline circulating ACE2 activity. The overall survival at 4 years of follow-up was assessed and patients were classified according to the cut-off value found in the ROC curve analysis. This value (24.9 RFU/μL/h) resulted from the direct association between atheromatosis at 2 years of follow-up and baseline ACE2, suggesting it as a good cut-off value for CV outcomes at 4 years of follow-up. The results confirmed a significant higher CV mortality in CKD3-5 patients with ACE2 levels above the cut-off value as compared to patients with low ACE2 levels. Cox regression analysis further confirmed the association of circulating ACE2 activity with non-fatal and fatal CV outcomes over the 4 years of follow-up.

Soro-Paavonen et al. found that ACE2 activity was negatively associated with estimated GFR in male and female patients with type 1 diabetes, suggesting that counter-regulatory mechanisms are activated in DN [167]. In experimental studies, diabetic ACE2 deficiency results in increased albuminuria in the context of diabetes [128,227,228], suggesting that ACE2 may provide renal protection. However, to our knowledge, there are no previous studies exploring the association between circulating ACE2 activity and renal function progression. We did not find any significant difference in patients that doubled serum creatinine or declined GFR at 24 months of

follow-up versus stable patients. The lack of significant differences might be related to the low number of patients with renal endpoints at 2 years of follow-up. Further studies with long-term follow-up will help to elucidate ACE2 as a biomarker of renal progression.

B.Experimental study

NOD mice were used as a model of DN for the experimental study. This animal model mimics human type 1 diabetes with destruction of pancreatic β cells. Features of DN, such as albuminuria and renal lesions have been reported in this animal model [87,88]. For this study, female NOD/ShiLtJ were used, because of the marked sex difference in the incidence of diabetes, being of 80% in females and less than 20% in males at 30 weeks of age [85]. NOR/LtJ females were used as control group, because NOR mice develop the same immunological phenotype as NOD mice, but without developing type 1 diabetes [229].

ACE2 activity has been shown to be altered in DN. The enzymatic activity and protein expression of ACE2 in STZ and in db/db mice are increased in renal cortex [128,166]. By contrast, ACE has been found to be decreased in the whole kidney cortex [128,165,230]. In agreement, previous studies by our group have shown similar results in NOD mice, where serum and urine ACE2 activity were increased at early and late stages of the disease. Interestingly, glycemic control by insulin administration prevented the diabetes-induced increase in the serum and urine ACE2 activity [88]. In kidney cortex, ACE2 activity and expression were also increased in early and late stages. In addition, increased serum and urine ACE2 activity correlated with increased UAE and GFR, as early markers of kidney involvement in diabetes [88]. In agreement, in this work we have found that serum, urine and renal ACE2 activity were increased in NOD as compared to the non-diabetic mice. Furthermore, there was a downregulation of circulating ACE2 activity by paricalcitol administration without modification on blood glucose levels and blood pressure.

As expected, blood glucose levels were increased in diabetic mice without changes by paricalcitol or aliskiren administration. Blood pressure was also increased in diabetic mice, a finding previously reported by our group. Blood pressure was increased in the early stage of the disease in the diabetic group and it was markedly reduced after insulin administration [88]. In the present work, high-dose paricalcitol showed similar SBP values compared with untreated diabetic animals, while low-dose administration tended to decrease it without statistical significance. To our knowledge, modulation of blood pressure by paricalcitol administration has not been reported neither in experimental studies [202,231] nor in human studies [232]. As expected, mice given aliskiren exhibited a significant decrease in SBP compared with non-treated diabetic mice. These results are in concordance with previous studies

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in animals and humans [193,233–235]. Thus, modulation of paricalcitol in circulating or renal ACE2 activity seems to be independent of blood pressure control.

Previous studies in experimental animal models have investigated the renoprotective effect of paricalcitol administration in diabetic kidney disease [236,237]. In humans, microalbuminuria is a major risk factor for progressive renal function decline in DN and is though to be the first step in an inevitable progression to proteinuria and renal failure [52,53,55]. Thus, reduction of albuminuria is a major target for renoprotective therapy in DN. Zhang et al. demonstrated a slight reduction in ACR levels in monotherapy with paricalcitol or losartan [236]. Moreover, combination therapy leads to significant reduction in albuminuria in STZ-diabetic mice. Consistently, the combination therapy of paricalcitol and losartan normalized the structure of the glomerular filtration barrier, preventing GBM thickening and podocyte effacement [236]. In a recent study in STZ-diabetic rats, treatment with paricalcitol and aliskiren lowered ACR compared to untreated diabetic rats [237]. In contrast, our study shows that paricalcitol administration either as monotherapy or in combination with aliskiren was unable to prevent albuminuria in the NOD model. One limiting point would be the origin of the urine samples. Given that protein excretion varies in the course of the day, collecting urine from metabolic cages, as Zhang et al. and Eren et al. performed in their studies, would be more informative than collecting the morning urine spot. In addition, the effect of paricalcitol in lowering albuminuria has been detected in diabetic mice with a more advanced DN (12 or 13 weeks of diabetes) as compared to our mice (21 days of diabetes). Nonetheless, it has been previously reported that NOD mice at this early stage of the disease have increased GFR and glomerular size, mimicking the renal hypertrophy and hyperfiltration characteristic of early DN in human type 1 diabetes [88]. In agreement with our results, a recently published study that explored the potential anti-inflammatory effects of VDR activation at different doses, demonstrated that paricalcitol was not able to reduce proteinuria in STZ-diabetic rats [238]. In addition, a human study enrolling type 2 diabetes and CKD patients was unable to show significant reductions in albuminuria by paricalcitol treatment [239]. The same pattern was observed in a large randomized clinical trial, where low-dose paricalcitol did not have an antiproteinuric effect in patients with DN, and the high-dose paricalcitol lowered residual albuminuria [240]. Importantly, although the high-dose paricalcitol was proposed as a novel approach to lower residual albuminuria, it was also associated with higher dropout rate owing to adverse effects than the low-dose treatment [240].

Another beneficial effect of paricalcitol is that it activates both antihypertrophic and antifibrotic activity. In an animal model of cardiac hypertrophy and interstitial fibrosis produced by chronic Ang II infusion, administration of paricalcitol led to a reduction in myocyte hypertrophy and left ventricular weight/total body weight ratio, a marker of cardiac hypertrophy. In addition, paricalcitol reversed the increase in collagen volume as a

consequence of Ang II infusion, suggesting an anti-fibrotic activity of paricalcitol in these animals [241]. Bae et al. demonstrated that paricalcitol treatment resulted in significant decrease in heart weight/body weight ratio in mice after myocardial infarction and reduced expression of fibrosis levels in the infarct zone [242]. Similarly, in uremic rats, cardiac weight and left ventricular weight are significantly reduced by paricalcitol treatment to values similar to the control rats, indicating prevention of cardiac hypertrophy [243,244]. Accordingly, in our model, animals given paricalcitol showed significant reductions in heart/body weight ratios compared with other diabetic animals. Furthermore, VDR activators or antagonists have shown to reverse or prevent the hypertrophic phenotype in various models of experimental hypertension such as Dahl S rats [245], spontaneously hypertensive rats [246], and heart failure-prone spontaneously hypertensive rats [247].

Regarding the RAS components, we found that plasma renin activity was increased in NOD mice and aliskiren treatment significantly lowered it. Human studies in CKD patients with hypertension and studies in an animal model of unilateral ureteral obstruction showed a reduction of plasma renin activity by aliskiren [248,249]. In the animal study by Choi et al., reduction of plasma renin activity was accompanied by an increase in renal mRNA expression of renin [249]. Similar results were found in mice with DN, where treatment with aliskiren significantly increased mRNA renin levels [250]. In our work, aliskiren treatment resulted in increased renal mRNA and protein expression of renin. We have also shown that paricalcitol significantly decreased mRNA expression and juxtaglomerular protein expression of renin. In concordance, paricalcitol significantly reduced renal renin expression at both gene and protein level in spontaneously hypertensive rats [246]. Same results were found in C57/BL6 mice in which paricalcitol elicited dose-dependent reductions in renin gene expression [205]. It has also been previously demonstrated that vitamin D stimulates plasma renin activity [251–253]. In our work, we have found a slight but not significant increase in plasma renin activity by high-dose paricalcitol treatment. Different results regarding plasma renin activity could result from different dose treatment with paricalcitol, being higher in those animal models in which a significant increase in plasma renin activity was achieved.

Renal ACE activity was decreased in diabetic mice as compared to controls, while serum ACE activity was increased. These results are in agreement with previous studies in which ACE activity was decreased in kidney cortex of db/db and STZ mice [166] and serum ACE activity was increased in STZ rats [254]. Administration of high-dose paricalcitol in NOD mice resulted in increased renal ACE activity and *Ace* gene expression. To our knowledge, there are no previous studies evaluating the effect of paricalcitol in ACE activity and expression.

We found a significant decrease in renal cortical Ang II in diabetic animals treated with paricalcitol and aliskiren in combination. Similar results have been

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observed in hypertensive Ren-2 rats, in which aliskiren treatment resulted in decreased Ang II concentration [255]. In our study, we have shown an increase in the Ang 1-7/Ang II ratio in treated diabetic animals that reached statistical significance with paricalcitol and aliskiren in combination.

The increase in serum ACE2 activity in diabetes is well known [88,166,227,256]. Our study describes the effects of paricalcitol on ACE2 activity. Interestingly, compared with non-treated diabetic animals, paricalcitol results in significantly decreased circulating ACE2 activity. Although the source for circulating ACE2 is not currently known, different studies have hypothesized that ACE2 may be actively shed from the cell surface through metalloproteases such as ADAM17 [141,257]. Recent studies have explored the relationship between ACE2 and ADAM17 in experimental DN models [142–144]. In diabetic Akita mice higher urinary ACE2 levels were associated with increased renal ACE2 and ADAM17 protein expression [143]. Salem et al. proposed that elevated levels of urinary ACE2 may be due to a rise in ectodomain shedding of renal ACE2 mediated by ADAM17 in the tubular membrane. Additionally, the molecular mass of urinary ACE2 was lower (70 kDa) than the molecular mass of kidney ACE2 (90 kDa), suggesting that the soluble form of ACE2 is the shed fragment of the membrane-bound ACE2 [143]. Insulin treatment normalized hyperglycemia in diabetic Akita mice and decreased both urinary and renal ACE2 protein expression and activity and ADAM17 protein expression [143]. Similar results were found by Chodavarapu et al. who showed that shedding of renal ACE2 into urine was increased in diabetic mice and that ADAM17 colocalized with tubular ACE2 in diabetic kidney [142]. They also observed a significant decline in blood glucose levels by rosiglitazone treatment, which also attenuated urinary ACE2 protein excretion and activity and renal ADAM17 in db/db mice [142]. Daily exercise training in db/db mice was also associated with blood glucose control and with a significant decrease in renal ADAM17 protein levels and ameliorated renal pathologies [144]. However, none of these studies describe a pharmacological intervention against ADAM17. The direct effect of paricalcitol in inhibiting this *sheddase* has been studied in the context of renal ostedystrophy [146]. Vitamin D inhibition of TACE expression and activity provides a potential mechanisms for the renoprotective actions of vitamin D metabolites, as increases in renal TACE expression have been reported to cause TGFα-driven renal lesions upon nephron reduction, ischemia or prolonged exposure to Ang II in mice [146]. Recently, Morgado-Pascual et al. showed that pre-incubation of tubular epithelial cells with paricalcitol downregulates ADAM17 gene overexpression to control values, suggesting that the anti-inflammatory properties of paricalcitol are, at least in part, mediated by inhibition of the ADAM17/TGF-α/EGFR pathway and downstream signals [147].

Our study indicates a direct involvement of paricalcitol in the modulation of circulating ACE2 activity in animals in which blood glucose is pathologically elevated. At the renal level, ACE2 protein increases in diabetes and further elevation was observed in paricalcitol-treated diabetic mice. This increase was

coupled with higher levels of gene expression in treated diabetic mice. In diabetic animals, renal tubules depicted high levels of ACE2 enzymatic activity that may increase when paricalcitol is administered. Activity detection was unable to show differences between treated and untreated animals. However, these differences became visible when analyzing ACE2 protein expression by Western blot and gene expression by real-time PCR. Diabetic animals treated with paricalcitol expressed higher levels of protein and gene in renal cortex than non-treated diabetic animals. These results suggest that the increase in ACE2 protein expression within the kidney cortex may be related to the increase in ACE2 mRNA levels in paricalcitol-treated animals. Regarding renal ADAM17, we observed a significant increase in ADAM17 in diabetic animals. As reported by Morgado-Pascual et al. [147], we found that high-dose paricalcitol resulted in decreased ADAM17 renal content to control levels, suggesting a direct effect of paricalcitol in modulating ADAM17.

To check the effect of paricalcitol within renal tubules, *in vitro* assays with MTC cells were performed. The studies in cultured cells showed that tubular cells treated with paricalcitol had higher levels of ACE2 protein expression, again indicating this protective role of paricalcitol in the diabetic milieu. Interestingly, Xiao et al. demonstrated that high glucose stimulated proximal tubular cell ADAM17 activity and led to increased ACE2 shedding [145]. Previous studies have also shown a protective effect of insulin administration to the podocyte by increased ACE2 expression, which was associated with decreased fibrosis markers and podocyte apoptosis [111,258]. Our results indicate that paricalcitol exerts its renoprotective effects in terms of decreasing renal content of ADAM17 and thus decreasing circulating ACE2 activity and keeping high levels of ACE2 in tubular cells. Interestingly, this effect is observed without changes in the glycemic status.

The activation of RAS and the oxidative stress caused by hyperglycemia are major mediators of development and progression of DN. In this regard, nitrotyrosine staining is increased in renal biopsies from DN patients, indicating that nitrotyrosine may be involved in the development of renal lesions in these patients [259]. Subsequent studies have further confirmed increased serum and kidney levels of nitrotyrosine in patients with DN and diabetic animal models [260,261]. In the context of diabetes, *in vivo* and *in vitro* studies have demonstrated an increase in H_2O_2 levels [262,263]. Thus, in our study we have analyzed the effect of paricalcitol in modulating oxidative stress by means of quantifying plasma H_2O_2 levels and the immunolocalization of nitrotyrosine as an indicator of nitric oxide-dependent oxidative stress. In this regard, we have shown that administration of high-dose paricalcitol resulted in reduction of circulating H_2O_2 and renal nitrotyrosine levels. Similarly, a recent study in STZ diabetic rats described a reduction in oxidative stress levels by calcitriol treatment [264]. Same results were found in uremic rats and in contrast-induced nephropathy rats treated with paricalcitol [265,266], as well as in hemodialysis patients where oxidative

stress levels were significantly decreased after three months on paricalcitol treatment [267].

C. General discussion

C.I. Circulating ACE2 and kidney disease

CV disease is the leading cause of morbidity and mortality in patients with CKD [32]. Few studies have examined the activity of circulating ACE2 in humans (Table 34). Soro-Paavonen et al. showed that type 1 diabetes patients with micro- or macrovascular disease displayed a significant increase in serum ACE2 activity when compared with controls or with a diabetic cohort without albuminuria [167]. In kidney transplant patients, our group studied circulating ACE2 activity and found that it positively correlated with age, graft function, glycosylated hemoglobin, creatinine and liver function parameters. Furthermore, circulating ACE2 activity was significantly increased in kidney transplant patients with ischemic heart disease [160]. To date, there is only one study analyzing circulating ACE2 activity in CKD patients. Plasma ACE2 activity was lower in hemodialysis patients when compared with pre-dialysis patients or renal transplant patients. The authors suggest that plasma ACE2 may increase early in the course of CKD, and be followed by a relative fall as CKD becomes established, with a further fall with an ESRD [168]. In agreement we initially found circulating ACE2 activity decreased in dialysis patients, however, when paired case-control studies were performed, differences among CKD patients were not observed. Thus, the initial decrease in ACE2 we saw in dialysis patients may be related to the different clinical characteristics of study cohorts, especially age. Roberts et al. also showed that in patients undergoing dialysis, ACE2 activity was significantly increased in males as compared to females, which was in concordance with other authors, including us [167,168]. In the present study we have shown that circulating ACE2 activity was increased in male CKD patients as compared to females.

Several experimental studies have also assessed circulating ACE2 activity (Table 34). Tikellis et al. reported a 2-fold increase in plasma ACE2 activity in STZ-induced diabetic male mice [227]. In agreement, Riera et al. demonstrated that serum and urine ACE2 activity were increased in the NOD mouse model of diabetes, both at early and late stages of the disease. Yamaleyeva et al. also found increased circulating ACE2 activity in early-onset diabetes after STZ administration in hypertensive mRen2.Lewis rats [256]. In rats with subtotal nephrectomy plasma ACE2 activity has been reported to be increased, and led to a 50% decrease in renal ACE2 activity [268]. In concordance, our experimental study also showed an increase in serum ACE2 activity in diabetic mice.

Table 34. Summary of human and experimental studies on circulating ACE2 activity in kidney disease.

	Study	Subjects / Animal model	Sample type	Circulating ACE2 findings
Human studies	**Soro-Paavonen et al. (2012)**	Type 1 diabetes (n=859) and healthy controls (n=204)	Serum	Increased in diabetic patients with micro- or macroalbuminuria Increased in males
	Soler et al. (2012)	Kidney transplant (n=113)	Serum	Increased in kidney transplant patients with ischemic heart disease Increased in males
	Roberts et al. (2013)	Predialysis CKD (n=59), hemodialysis (n=100), kidney transplant (n=89) and non-contemporaneous controls (n=18)	Plasma	Increased in CKD patients Increased in males
	Anguiano et al. (2015)	CKD3-5 (n=1458), CKD5D (n=546) and controls (n=568)	EDTA-plasma	Increased in males, older age and diabetic patients Associated with atheromatosis progression at 2 years of follow-up Associated with CV outcomes at 4 years of follow-up
Animal studies	**Tikellis et al. (2008)**	Diabetic (STZ induction) and control C57Bl6 male mice	Serum	Increased in diabetic mice
	Velkoska et al. (2010)	Subtotal nephrectomized and sham female Sprague-Dawley rats	Plasma	Increased after acute reduction in renal mass
	Yamaleyeva et al. (2012)	Diabetic (STZ induction) and control hypertensive mRen2.Lewis rats	Serum	Increased in hypertensive rats with early-onset of diabetes
	Riera et al. (2014)	NOD and NOR female mice	Serum	Increased in NOD mice
	Anguiano et al. (2015)	NOD and NOR female mice	Serum	Increased in NOD mice

Abbreviations: STZ, streptozotocin; NOD, non-obese diabetic mouse; CKD, chronic kidney disease.

Results from our human and experimental study, together with previous results from other groups, suggest that the increase in circulating ACE2 activity might be a mechanism to attenuate an increase in circulating Ang II. In addition, Soler et al. demonstrated an increase in circulating ACE2 activity in kidney transplant patients with ischemic heart disease [160], confirming

previous results from others, in which circulating ACE2 activity was associated with CV disease [155–159]. In our study, we have also confirmed an association between baseline circulating ACE2 and atheromatosis progression at 2 years and CV outcomes at 4 years of follow-up in CKD patients. Of note, that the population analyzed had no previous history of CV disease, suggesting circulating ACE2 as an important biomarker of CV risk in kidney disease patients.

C.II. ACE2 modulation

Given the importance of both circulating and renal ACE2 in kidney disease, several groups have studied kidney injury after genetic and pharmacological ACE2 modulation. In this regard, the ACE2 inhibitor MLN-4760 has been used in murine models. Tikellis et al. demonstrated a reduction in plasma ACE2 activity, cortical ACE2 activity and cortical levels of Ang 1-7 by treatment with MLN-4760 [227]. Accordingly, Soler et al. demonstrated a 90% reduction in renal ACE2 activity from STZ mice treated with MLN-4760. Furthermore, inhibition of ACE2 was associated with increased albuminuria, glomerular mesangial expansion and vascular thickness [230]. In db/db mice, inhibition of ACE2 by MLN-4760 resulted in a significant increase in albumin excretion, which by 24 weeks of age, was approximately 3-fold higher as compared with vehicle-treated db/db mice [128]. DX600 has been also studied as a potential ACE2 inhibitor. In proximal tubular cells, addition of DX600 completely blocked conversion of Ang I to Ang 1-7 [125]. Studies in vascular cells showed that DX600, by inhibiting ACE2, significantly accelerated the Ang II-mediated proliferation [269]. In cardiofibroblasts, pharmacological inhibition of ACE2 with DX600 largely aggravated Ang II-induced enhancement of superoxide production and NADPH oxidase activity [270].

Initial studies focused on ACE2 KO mice, demonstrated a significant increase of Ang II levels in the kidneys, hearts and plasma [119,271,272], which was associated with Ang II-dependent hypertension, severe albuminuria and increased glomerular volume, mesangial matrix expansion and GBM thickness [271,272]. ACE2 KO mice showed an age-dependent development of glomerular mesangial expansion, hypothesizing that the link between ACE2 deletion and renal injury may be the chronic exposure to increased circulating and tissue Ang II levels [133]. This hypothesis was confirmed by administration of an ARB to ACE2 KO mice, which prevented glomerular injury and development of glomerulosclerosis and albuminuria [133]. ACE2 KO mice crossed with the type 1 diabetes model Akita mice, exhibited a 2-fold increase in UAE, increased mesangial matrix scores and GBM thickness [228]. In addition, although kidney levels of Ang II were not increased in the diabetic mice, treatment with an ARB reduced UAE in ACE2 KO mice, suggesting that acceleration of glomerular injury was Ang II-mediated [228]. Recently, a study in ApoE/ACE2 double KO mice associated elevated Ang II levels with increased expression of pro-inflammatory cytokines

and chemokines, including TNF-α and IL-6 [273]. All these findings suggest a role of ACE2 in regulating Ang II-mediated kidney injury.

Therefore, agents that increase ACE2 activity at the tissue level have been proposed for chronic therapy in kidney disease. The group of Hernandez-Prada et al. found a compound (xanthenone) capable of enhancing cardiac ACE2 activity by approximately 2-fold. Interestingly, acute intravenous injections of xanthenone resulted in a decrease in blood pressure and a reversal of cardiac and renal fibrosis in a spontaneously model of hypertension [274]. Subsequent studies in pregnant Sprague-Dawly rats also showed a reduction in urinary protein excretion and markers of endothelial activation [275]. Overexpression of ACE2 by lentiviral vector constructs has been also studied in cardiac disease and acute lung injury. In the initial study by Huentelman et al., overexpression of ACE2 demonstrated a protection against the development of Ang II-induced myocardial fibrosis beyond a normalization of SBP [154]. *In vitro* and *in vivo* studies also confirmed that ACE2 protein could be sustainably overexpressed, resulting in anti-inflammatory and anti-apoptotic effects [276,277]. Local overexpression of ACE2 by adenoviral vectors has been also studied in a model of atherosclerosis and showed greater conversion of Ang II to Ang 1-7, decreased Ang II and increased Ang 1-7 levels, and reduced ACE activity [223]. Further studies in which recombinant ACE2 was used, have demonstrated an increase in nephrin levels and renal ACE2 and Ang 1-7/Ang II ratio [273]. Wysocki et al. studied the effect of recombinant ACE2 in a model of Ang II-induced hypertension and confirmed that it prevented hypertension as a result of ACE2-driven Ang II degradation within the circulation [278]. To our knowledge there is only one study analyzing the effect of recombinant ACE2 in DN. Akita mice treated with recombinant ACE2 showed a reduction in albumin excretion, glomerular hypertrophy and mesangial matrix expansion, confirming that modulation of angiotensin peptide metabolism and its downstream effects can attenuate DN. In addition, the study suggested that blockade of Ang 1-7 signaling with a Mas receptor peptide antagonist limited the protective effect of recombinant ACE2 *in vitro* [134].

RAS blockade by ACE inhibitors, ARBs and aldosterone antagonists also increases ACE2 enzymatic activity and protein [163,279–284]. Ferrario et al. showed that ARB or ACE inhibitors significantly increased cardiac ACE2 gene expression [279]. In kidney, ACE2 gene expression has been also found to be increased by treatment of animals with an ARB [280]. This results were confirmed in the study by Jessup et al., in which treatment with ARB or ACE inhibitor resulted in increased cardiac and renal ACE2 gene expression and enzymatic activity [163]. In an experimental model of myocardial infarction, left ventricular ACE2 activity and gene expression were significantly increased by treatment with an ACE inhibitor [281]. Aldosterone antagonists increase ACE2 enzymatic activity and gene expression in human monocyte-derived macrophages isolated from blood samples obtained from congestive heart failure patients [282]. A subsequent study showed increased ACE2 gene

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expression levels in the heart of hypertensive rats [283]. In the kidney, immunofluorescence studies revealed blunted ACE2 staining and protein expression in aldosterone-infused rats, suggesting that ACE2 expression may play an important role in aldosterone-induced kidney injury [284].

Modulation of ACE2 has been also demonstrated in the NOD mouse model, in which treatment with insulin resulted in a significant reduction in serum and urine ACE2 activity and a prevention of renal alterations such as glomerular enlargement and hyperfiltration [88]. To our knowledge no studies have assessed the modulation of ACE2 activity by treatment with vitamin D analogs. In our human study we have found that treatment with the vitamin D cholecalciferol was associated with reduced circulating ACE2 activity in dialysis patients. In addition, the experimental study confirmed a modulation of ACE2 by treatment with the vitamin D analog paricalcitol. This is the first study showing renoprotective effects of paricalcitol in terms of decreasing circulating ACE2 activity and increasing renal ACE2 expression, beyond lowering blood pressure and albuminuria. These results suggest that keeping high levels of ACE2 at kidney level and low levels of ACE2 within the circulation may enhance Ang II conversion into Ang 1-7 in the kidney, thus promoting anti-fibrotic, anti-proliferative and anti-inflammatory effects that may help to slow down the progression of kidney disease (Figure 50).
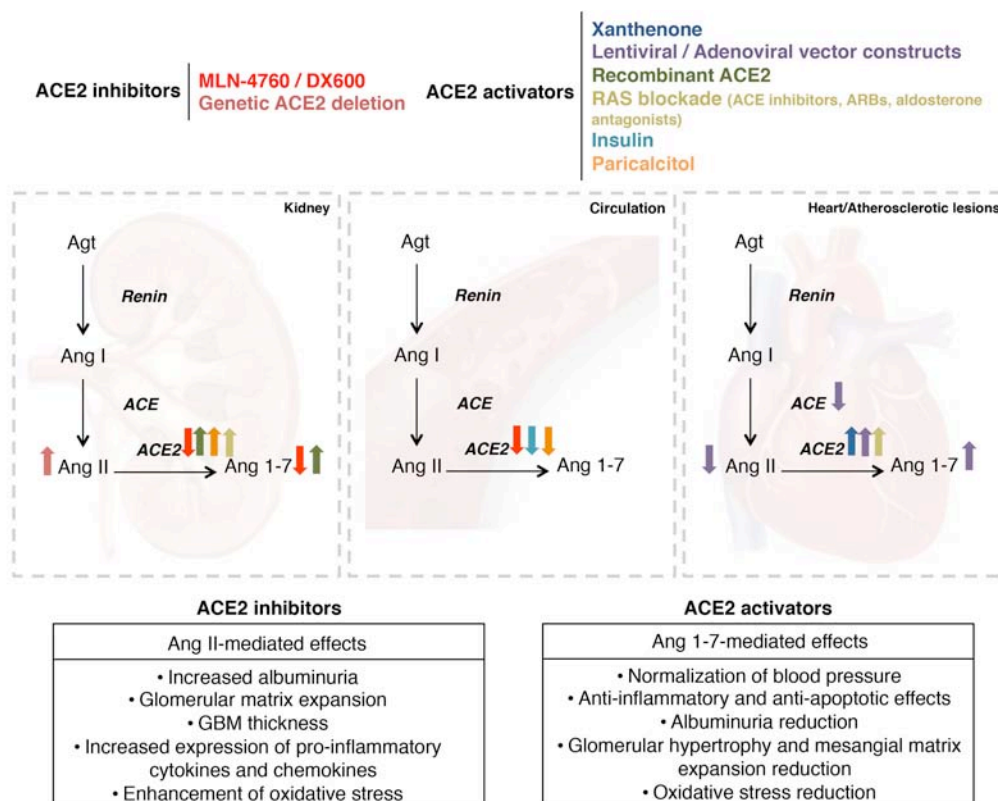
Figure 50. Schematic representation of ACE2 inhibitors and activators on modulating renin-angiotensin system and its downstream effects. As a consequence of direct ACE2 inhibition, Ang II-mediated injury occurs, while activation of ACE2 results in opposite effects to those mediated by Ang II.

C.III. ACE2/ADAM17 pathway

Donogue and Tipnis, who simultaneously identified ACE2, confirmed the proteolytic cleavage of ACE2 from the cell surface and the secretion of a soluble form of ACE2 lacking the transmembrane and cytosolic domains [117,118]. A few years later, Lambert et al. demonstrated in *in vitro* studies that ACE2 underwent proteolytic shedding, releasing an enzymatically active ectodomain [141]. Furthermore, this study evidenced a direct involvement of ADAM17 in the stimulated ACE2 shedding [141]. It has been also proposed that shedding of ACE2 also occurs *in vivo* in humans [155,156,285]. Western blot analysis have indicated that most of the immunoreactive ACE2 is smaller than the full-length enzyme, and thus is likely to result from proteolytic cleavage [156].

In vitro studies have demonstrated an up-regulation of ADAM17 in high-glucose conditions that translated to increased ADAM17 activity [145,286–288]. Higher ADAM17 activity in cell lysates was accompanied by an increase in ACE2 activity in the media [145]. In mesangial cells treated with high-glucose, ADAM17 was normalized by administration of a specific ADAM17 inhibitor (TAPI2), thus regulating the profibrotic response to glucose in these cells [287,288]. *In vivo* studies also confirmed that diabetes was associated with increased renal ADAM17 expression that was normalized as a consequence of blood glucose reduction by antidiabetic treatments or daily exercise [142–144]. Accordingly, our work further demonstrates that circulating ACE2 activity is increased in diabetic conditions. In addition, diabetic NOD mice exhibited increased ADAM17 renal content, suggesting that the action of renal ADAM17 may be an important source of circulating ACE2 activity (Figure 51). Infusion of Ang II in mice also demonstrated an enhancement in ADAM17 expression in the vasculature, cardiac myocytes and renal tubules [289,290].

Several studies have demonstrated ADAM17 immunoreactivity in experimental and human atherosclerotic lesions [291][292]. Canault et al. demonstrated that ADAM17 present in the surface of microparticles isolated from human atherosclerotic lesions stimulated the shedding of the ADAM17 substrates TNF and TNF receptor [293]. Monocytes from subjects characterized by increased insulin resistance and atherosclerosis exhibited reduced mRNA levels of the ADAM17 inhibitor TIMP3 [294]. Thus, down-regulation of TIMP3 may increase ADAM17 activity, leading to increased shedding of its substrates. Results from these studies, together with our findings of an independent association between increased baseline circulating ACE2 and the number of territories with plaques, may indicate an involvement of ADAM17 in the shedding of ACE2 to circulation and its association with atheromatosis progression in CKD3-5 patients from the NEFRONA Study. In a recent study, baseline ADAM17 activity was evaluated by dosing its four main circulating substrate levels (soluble TNF receptor 1, soluble IL-6 receptor,

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soluble VCAM-1 and soluble ICAM-1). Increased circulating levels of ADAM17 substrates were associated with a significant higher rate of second CV events [295]. The authors hypothesized that, although ADAM17 protease activity was not explored directly *in vivo*, it is conceivable that the increase of its substrates in the circulation depends from increased enzymatic activity at local inflammatory sites. In this regard we have also found a direct association of the ADAM17 substrate, ACE2, with CV events at 4 years of follow-up. The hazard ratio was lower than that obtained in the previously mentioned study by Rizza et al. This might be explained by the different population studied, since patients included in the NEFRONA Study have no previous history of CV disease and ACE2 was assessed alone as a biomarker of CV outcomes, while in the study by Rizza et al. patients have an established vascular atherosclerosis and a set of four circulating substrate levels was analyzed.

In our experimental study we have reported for the first time a direct modulation of paricalcitol on ADAM17 levels in a model of DN (Figure 51). We observed a decrease in ADAM17 renal content accompanied by decreased serum ACE2 activity and increased ACE2 positive tubule staining in MTC cells. Similarly, in CKD5D patients from the human study we showed that cholecalciferol, one of the five forms of vitamin D, was directly associated with decreased circulating ACE2 activity. In this regard, it has been reported that homozygous mutant mice lacking the VDR gene develop cardiac hypertrophy, suggesting that vitamin D supplement may be beneficial to the CV system [188,202,203]. Furthermore, in the NEFRONA population, low levels of 25OH vitamin D were associated with atheromatosis progression [44]. Therefore, the reduction in circulating ACE2 activity and the increase in renal ACE2 expression levels as a consequence of paricalcitol administration may be, at least in part, mediated by the reduction of renal ADAM17 levels, and thus, of its shedding activity (Figure 51). This modulation of ACE2 may enhance degradation of tissue Ang II to Ang 1-7, promoting anti-fibrotic, anti-proliferative and anti-inflammatory effects.
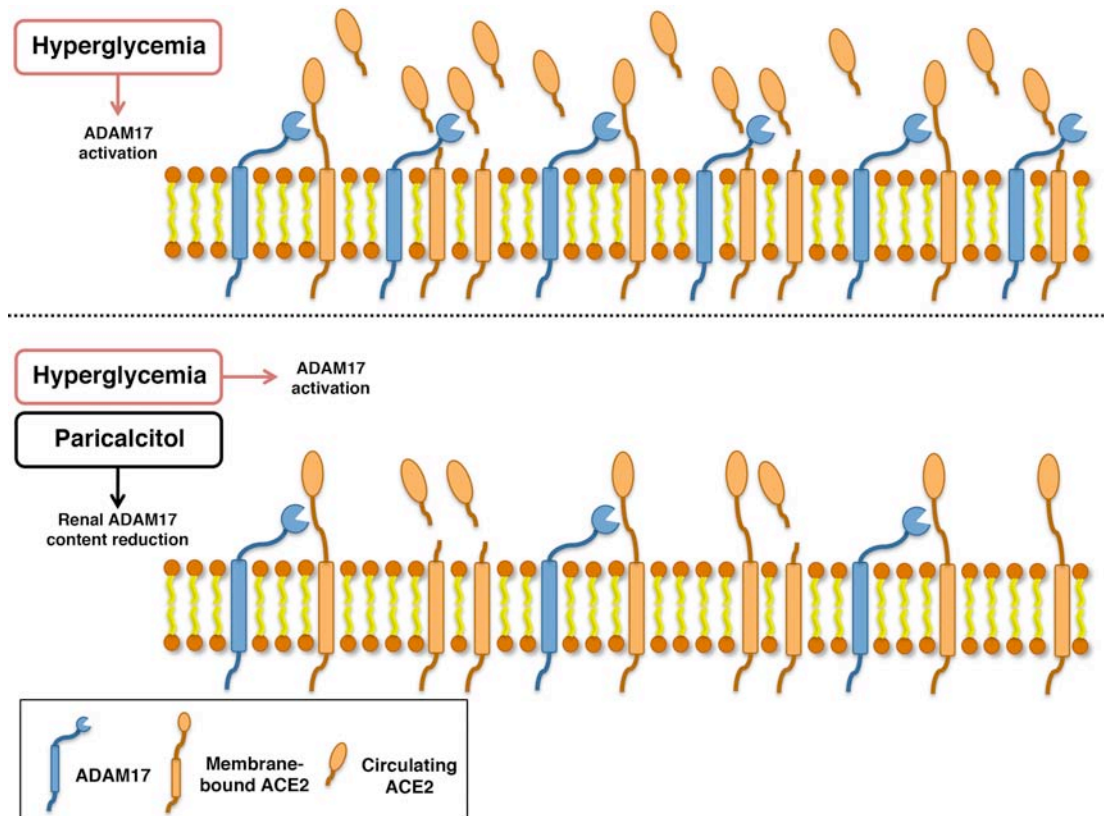
Figure 51. Effect of paricalcitol in ACE2 shedding. In the context of diabetes, ADAM17 activation results in increased ADAM17 activity, and thus, in increased shedding of ACE2 from the cell membrane. Therapeutic intervention with paricalcitol leads to a reduction in renal ADAM17 content, thus reducing circulating ACE2 activity and increasing renal ACE2 expression.

In summary, the association observed between increased circulating ACE2 activity and atheromatosis progression and diabetes, and the modulation of its activity by administration of paricalcitol, suggests that increased circulating ACE2 activity in kidney disease patients may be used as a powerful biomarker for disease progression.

VII. CONCLUSIONS

VII. CONCLUSIONS

1. Circulating ACE2 and ACE activities can be detected in EDTA-plasma samples by the addition of a specific concentration of zinc chloride.

2. Baseline circulating ACE2 activity is decreased in CKD3-5 patients and CKD5D patients without previous history of CV disease, while circulating ACE activity is increased.

3. Baseline circulating ACE2 activity correlates with classical CV risk factors, namely male gender, older age and diabetes in CKD3-5 patients. In this population, circulating ACE activity correlates with male gender and older age.

4. The prospective study demonstrates an independent and direct association between baseline ACE2 activity and a higher risk for silent atherosclerosis progression at 2 years of follow-up. This suggests that ACE2 activity may serve as a biomarker to predict CV risk before a CV disease is established.

5. The prospective study also shows a direct association between baseline circulating ACE2 and a higher risk of CV outcomes at 4 years of follow-up.

6. In the animal model of type 1 diabetes, paricalcitol modulates circulating and renal ACE2 activities and reduces oxidative stress.

7. In the experimental study, renal ADAM17 levels are increased in the context of diabetes, and paricalcitol administration results in a significant reduction in these levels.

8. *In vitro* studies in mouse tubular epithelial cells show that paricalcitol incubation increases ACE2 expression in a dose-dependent manner.

9. Increased levels of ACE2 in tubular cells and low levels within the circulation as a consequence of paricalcitol administration and probably due to its lowering effects in renal ADAM17 levels, may help to slow down the progression of DN.

VIII. LIMITATIONS AND FUTURE PERSPECTIVES

VIII. LIMITATIONS AND FUTURE PERSPECTIVES

A. Limitations

- In the human study, only CV and non-CV outcomes were reported at 4 years of follow-up, without any information on renal function and silent atherosclerosis parameters. Given that the NEFRONA Study was not designed for the study of circulating ACE2, but for the analysis of morbidity and mortality in CKD patients, the lack of more information at the end-point of the study did not allow us to identify baseline ACE2 as a possible biomarker of renal function and silent atherosclerosis at a more advanced follow-up of patients. Furthermore, patients included in the NEFRONA Study have no previous history of CV disease, resulting in a low number of events at the end of the study. Therefore, although circulating ACE2 activity may be used as a biomarker of atheromatosis progression, it is difficult to draw conclusions at a long-term follow-up.
- In the animal model of DN, urine collection may be a limiting point in the determination of albuminuria. Although there was a decrease in the levels of albuminuria in diabetic mice treated with paricalcitol it did not reach statistical significance, probably due to the high standard error observed. Collecting urine from metabolic cages instead from the morning urinary spot would be more informative.

B. Future perspectives

In this thesis circulating ACE2 activity has been proposed as a possible biomarker of silent atherosclerosis, and thus, of long-term CV outcomes in CKD. Furthermore, experimental studies in a model of DN have revealed paricalcitol as a modulator of renal and circulating ACE2 activities and of renal ADAM17 levels. Given the direct association between ACE2 and ADAM17 in kidney disease, future studies should be designed:

- To increase the follow-up study from the NEFRONA project to 6 years, to assess renal events, atheromatosis progression and CV outcomes.
- To further investigate the ADAM17/ACE2 axis in CKD patients by the study of circulating ADAM17 activity as another possible biomarker of CV outcomes.
- To analyze circulating ACE2 together with other possible biomarkers of CV disease (soluble tweak, phosphorous, and ADAM17 among others) in patients from the NEFRONA project. With this strategy, the development of a panel biomarker for CV disease in CKD patients is feasible.
- To deepen in the mechanisms implicated in the modulation of ACE2 and ADAM17 in experimental models of DN.
- To study the possible pathways activated in tubular cells and experimental models of DN as a consequence of the administration of paricalcitol.

IX. REFERENCES

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