

Original Article

## Circulating angiotensin-2 levels increase with progress of chronic kidney disease

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### Abstract

**Background.** Angiotensin-2 (Ang-2) is an antagonistic ligand of the endothelial-specific Tie2 receptor. Patients on dialysis have markedly elevated Ang-2 levels, and those correlate with their atherosclerotic burden. The aim of the current study was to investigate the relationship between the circulating levels of Ang-2 and renal function throughout all stages of chronic kidney disease (CKD). In addition, we aimed to detect a potential link between the nitric oxide (NO) synthase inhibitor asymmetric dimethylarginine (ADMA) and the Ang-2 levels.

**Methods.** Glomerular filtration rate (GFR) was assessed by the inulin clearance technique ( $\dot{V}$ GFR) and compared to serum Ang-2 (immunoluminometric assay) and ADMA levels (liquid chromatography–electrospray tandem mass spectrometry) in 44 untreated non-smokers at the different stages of CKD 1–4. Ang-2 was also measured in 19 patients on dialysis (CKD stage 5). In addition, the Ang-2 and  $\dot{V}$ cGFR (cystatin C) measurements were taken in 15 healthy individuals before and 72 h after kidney donation.

**Results.** The median Ang-2 levels steadily increased across the following groups: healthy controls: 0.77 (0.32–1.08) ng/mL; CKD 1: 0.83 (0.67–1.09) ng/mL; CKD 2: 0.93 (0.74–1.15) ng/mL; CKD 3: 1.13 (0.87–1.49) ng/mL; CKD 4: 1.75 (1.23–2.61) ng/mL; and CKD 5: 4.87 (3.22–7.59) ng/mL, respectively (non-parametric ANOVA  $P < 0.0001$ ). Ang-2 was associated with the degree of CKD as evidenced by an inverse correlation with the  $\dot{V}$ GFR ( $r = -0.509$ ,  $P < 0.0001$ ) and positive correlations with homocysteine ( $r = 0.365$ ,  $P = 0.015$ ) and phosphate ( $r = 0.53$ ,  $P < 0.0001$ ). Additionally, Ang-2 correlated with the ADMA levels ( $r = 0.35$ ,  $P = 0.01$ ). We detected a close inverse correlation between the mean changes in GFR and circulating Ang-2 at 72 h after kidney donation ( $r = -0.54$ ,  $P = 0.03$ ).

**Conclusions.** Circulating Ang-2, a putative marker and potential mediator of accelerated atherosclerosis, is inversely related to GFR and increases with advanced CKD. The correlation between Ang-2 and ADMA points towards the hypothesis that the ADMA-driven NO deficiency might trigger Ang-2 release and account for the Ang-2 increase in CKD patients.

**Keywords:** angiotensin; CKD; dialysis; kidney donation; nephrectomy

### Introduction

The global population of stage 5 chronic kidney disease (CKD) patients was estimated to have reached ~1.7 million, and continues to grow at a significantly higher rate than the world population [1,2]. CKD patients (even in the lower stages) are more likely to develop cardiovascular (CV) disease [3] and to die from it than people with a normal kidney function [4]. In CKD, the classical CV risk factors sometimes not only fail to predict survival and CV burden but show a reverse epidemiology [5]. Some new CV risk factors are more powerful indicating CV disease or endothelial dysfunction in CKD [6–11]. Moreover, some of these new markers [e.g. asymmetric dimethylarginine (ADMA), neuropeptide Y, visfatin] may also play an important role as mediators of CV disease in CKD [11–13].

Among the members of the angiotensin (Ang) family Tie2 receptor and its ligands, Ang-1 and Ang-2 have attracted much attention as the factors related to angiogenesis and inflammation [14,15]. The Ang/Tie system tightly controls the endothelial phenotype during angiogenesis and vascular inflammation in a unique and non-redundant fashion [16,17]. Produced by vascular smooth muscle cells and pericytes, Ang-1 stabilizes the development of newly

**Table 1.** Clinical parameters of healthy control subjects and patients with CKD stage 1–5

Parameter	Healthy Controls	Patients					<i>P</i> -value
		CKD 1	CKD 2	CKD 3	CKD 4	CKD 5/dialysis	
Number of patients	29	16	9	8	10	14	–
Gender (male/female)	16/13	10/6	3/6	4/4	6/4	9/5	–
Age (years)	57.6 ± 12.5	41.25 ± 9	47.3 ± 8	45.1 ± 8	45.45 ± 9	57.8 ± 13	0.342
S-creatinine (mg/dL)	0.79 ± 1.08	1.05 ± 0.1	1.68 ± 0.3	2.32 ± 0.7	4.45 ± 0.9	7.92 ± 3.08	<0.0001
$\dot{V}$ GFR (mL/min/1.73 m <sup>2</sup> )	–	120 ± 15	70 ± 8	46 ± 12	24 ± 4	–	<0.0001
BMI (kg/m <sup>2</sup> )	25.2 ± 3.5	26.3 ± 3.9	26.5 ± 1.9	26.1 ± 3	26.4 ± 3.9	24.7 ± 4.2	0.996
MAP (mmHg)	92 ± 8	103 ± 10	108 ± 11	109 ± 6	111 ± 9	96 ± 12	0.257
Hcy (μmol/L)	–	10.9 ± 2.9	18.8 ± 4.6	17.2 ± 3.6	29.5 ± 11.6	35 ± 17.1	<0.0001
Cholesterolin (mg/dL)	–	195.3 ± 37	205.2 ± 24	192 ± 24	193 ± 40	131.2 ± 26	0.818
Triglycerides (mg/dL)	–	157.6 ± 50	153.6 ± 54	156.4 ± 59	166.1 ± 61	145.5 ± 51	0.755
Calcium (mmol/L)	–	2.24 ± 0.07	2.33 ± 0.09	2.3 ± 0.1	2.24 ± 0.16	2.12 ± 0.2	0.178
Phosphate (mmol/L)	–	0.89 ± 0.2	1.08 ± 0.23	1.15 ± 0.3	1.36 ± 0.23	2.09 ± 0.49	<0.0001
Ca × Ph product (mmol <sup>2</sup> /L <sup>2</sup> )	–	2 ± 0.47	2.5 ± 0.54	2.63 ± 0.7	3.05 ± 0.57	4.4 ± 0.3	<0.0001
iPTH (pmol/L)	–	4.9 ± 1.4	6.4 ± 1.5	23.6 ± 32.4	38.6 ± 18.3	54.7 ± 75.8	<0.0001
Ang-2 (ng/mL)	0.9 ± 0.68	1.0 ± 0.6	0.97 ± 0.27	1.32 ± 0.7	1.9 ± 0.8	6.1 ± 4.2	0.004

CKD subgroups were analysed by using non-parametric ANOVA test followed by *post hoc* Dunn's test for multiple comparisons. Values are expressed as mean ± SD or total numbers.

CKD, chronic kidney disease;  $\dot{V}$ GFR, inulin-measured glomerular filtration rate; BMI, body mass index; MAP, mean arterial blood pressure; Hcy, homocysteine; Ca × Ph product, calcium–phosphate product; iPTH, intact parathyroid hormone; Ang-2, angiotensin-2.

formed blood vessels by recruiting mural cells and promotes quiescence and structural integrity of mature vessels mainly by activation of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway [18]. In the adult vasculature, the constitutive Ang-1 expression and low-level Tie2 phosphorylation probably represents a control pathway to maintain vascular quiescence by anti-apoptotic and anti-inflammatory effects, thus protecting the endothelium from excessive activation by cytokines and growth factors.

Ang-2 is expressed in endothelial cells, where it is stored in granules, the so-called Weibel–Palade bodies (WPB) [19]. The release of Ang-2 upon activation of the endothelium with for instance thrombin, histamine or hypoxia disrupts the constitutive Ang-1/Tie2 signalling in a dose-dependent manner by preventing Ang-1 from binding to the receptor [17,20]. Consequently, the loss of Tie2 signalling destabilizes the endothelium and facilitates the angiogenic or inflammatory response to growth factors and cytokines [21]. Hence, Ang-2 functions as a dynamic autocrine-negative regulator of the quiescent resting endothelium [22].

Elevated levels of Ang-2 have been found in diabetes mellitus [23], arteriosclerosis [24,36], acute coronary syndrome [25], arterial hypertension [37,38], systemic lupus erythematosus [26], acute kidney injury [35], and sepsis [27,28]. We were recently able to show that circulating Ang-2 is also markedly elevated in patients on maintenance dialysis and that Ang-2 is closely associated with the extent of coronary heart disease (CHD) and peripheral arterial disease (PAD) [7]. However, our previous study lacks data on the association between Ang-2 and CKD 1–4. Based on the prior data, we hypothesized a pivotal, and probably indirect, role of the preserved renal function to maintain normal (low) circulating Ang-2 levels. Moreover, as a putative mediator of accelerated atherosclerosis in CKD, excess Ang-2 is probably not restricted to CKD 5 (i.e. maintenance dialysis) but should already start to in-

crease at the earlier CKD stages, an association that we found a decade ago for ADMA, the most potent endogenous inhibitor of nitric oxide (NO) [8]. Intriguingly, Ang-2 is released from WPB in states of NO deficiency. Based on these data from the literature, we speculated that the elevated Ang-2 levels in CKD patients might be due to the excess WPB exocytosis as a consequence of decreased NO production in the presence of high ADMA levels.

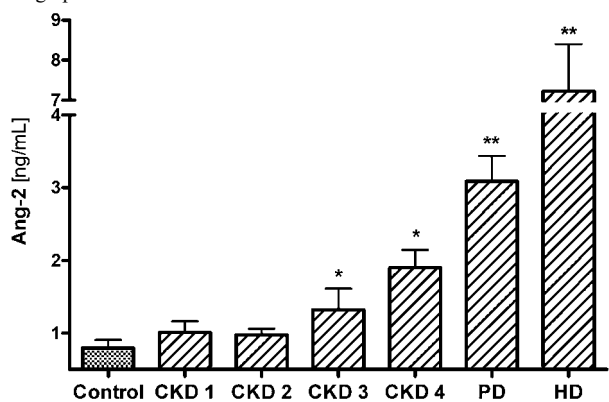
According to this sophisticated hypothesis, we aimed to investigate the relationship between Ang-2 and both ADMA levels and glomerular filtration rate (GFR) in CKD patients across stages 1–5 as well as after a unilateral nephrectomy in healthy kidney donors.

## Materials and methods

The local ethics committee approved the protocol, and all participants gave informed consent. In total, 78 study subjects and 29 apparently healthy controls participated in the study. The impact of renal function on the circulating Ang-2 levels was investigated in CKD patients [Kidney Disease Outcomes Quality Initiative (K/DOQI) stage 1–5,  $n = 63$ ]. To overcome the problem of between-subject effects in relation to the CKD stages (e.g. extent of prevalent micro-inflammation), the Ang-2 levels were longitudinally analysed after a unilateral nephrectomy in healthy kidney donors ( $n = 15$ ).

### CKD patients

A total of 63 (out of 233 eligible) adult Caucasians aged 45 (39–54) years with primary CKD due to (i) biopsy-proven immunoglobulin A glomerulonephritis (IgA GN,  $n = 34$ ) or (ii) adult polycystic kidney disease (ADPKD,  $n = 29$ ) confirmed by family history and ultrasound examination from CKD stage 1 to 5 were included in the study. Consequently, patients with presumable Ang-2 alterations due to any systemic endothelial activation (e.g. systemic lupus erythematosus [26] and vasculitis [29]) as well as diabetic patients were excluded. The five groups were matched with respect to age, gender and body mass index (BMI) (Table 1). The patients with CKD 1–4 had a stable renal function for at least 6 months prior to inclusion. Details of the inulin clearance method used in these patients have been published previously [30]. In all of these patients, a detailed history and a thorough physical examination, including electro-



**Fig. 1.** Bars chart (mean  $\pm$  SEM) of circulating Ang-2 levels in apparently healthy controls and patients stratified to CKD stages. Groups were compared by using non-parametric Kruskal–Wallis test followed by *post hoc* Dunn's test for multiple comparisons (\* $P < 0.01$ , \*\* $P < 0.0001$  compared to 'healthy controls'). Compared to healthy controls, Ang-2 levels were significantly elevated beginning with CKD stage 3. CKD stage 5 patients are subdivided according to treatment by HD or PD.

cardiogram, were obtained to exclude peripheral arterial disease (PAD) and coronary heart disease (CHD).

#### Healthy kidney donors

Blood samples from 15 healthy kidney donors [54 (48–59) years, 5 males and 10 females] without previous history of CHD, PAD, diabetes mellitus or prevalent CKD were taken at the initiation of surgery (shortly before the unilateral nephrectomy) and after 72 h post-surgery. The GFR was calculated by means of the cystatin C blood levels ( $cGFR$ ) using the following formula [31]:  $cGFR [mL \times min^{-1} \times (1.73 m^2)^{-1}] = 84.69 \times cystatin\ C (mg/L)^{1.680}$ .

#### Healthy controls

Twenty-nine apparently healthy non-smoking subjects ( $57.9 \pm 15.1$  years of age) were included as controls. The controls were matched with respect to age, gender and BMI to the groups of patients.

#### Sampling and measurements

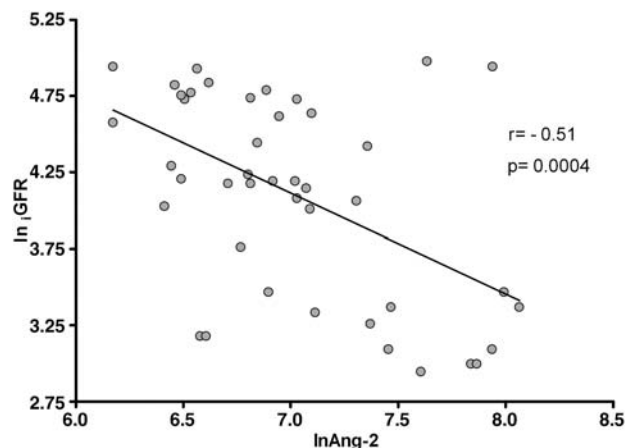
Blood pressure (BP) was measured oscillometrically with the patient in supine position in a quiet environment with an automated device (Dinamap, Critikon Co., Tampa, FL). The mean of three consecutive measurements 5 min apart after a rest of at least 20 min was taken for analysis. Blood samples for measurement of Ang-2, ADMA, creatinine, total homocysteine (Hcy), intact parathyroid hormone (iPTH) and total cholesterol concentrations were drawn in a supine position in the morning after at least 12 h of fasting [in haemodialysis (HD) patients 10 min before starting the mid-week dialysis session].

#### Quantification of circulating Ang-1, Ang-2 and ADMA

Plasma Ang-1 was measured by in-house enzyme-linked immunosorbent assay (ELISA) as previously described [32]. Plasma Ang-2 was measured by in-house immunoluminometric assay (ILMA) methodology as described previously [21–25]. In brief, recombinant human Ang-2, monoclonal Ang-2 AB and anti-Ang-2 AB were purchased from R&D Systems (R&D, Oxon, UK). The assay had a detection limit of 0.2 ng/mL. Inter-assay and intra-assay imprecision was 4.6% and 5.2%, respectively. The plasma concentrations of ADMA were measured by applying a recently developed liquid chromatography–mass spectrometry method described elsewhere [33].

#### Statistical analysis

Data are presented as median (25–75% percentile) unless otherwise stated. The CKD 1–5 subgroups were analysed using the non-parametric Kruskal–Wallis test followed by *post hoc* Dunn's test for multiple compar-



**Fig. 2.** Scatter plot showing the correlation of logarithmic (ln) angiotensin-2 (lnAng-2) level with the corresponding inulin-measured glomerular filtration rate (ln<sub>i</sub>GFR) in 44 stage 1–4 CKD patients (Pearson's correlation:  $r = -0.51$ ,  $P = 0.0004$ ).

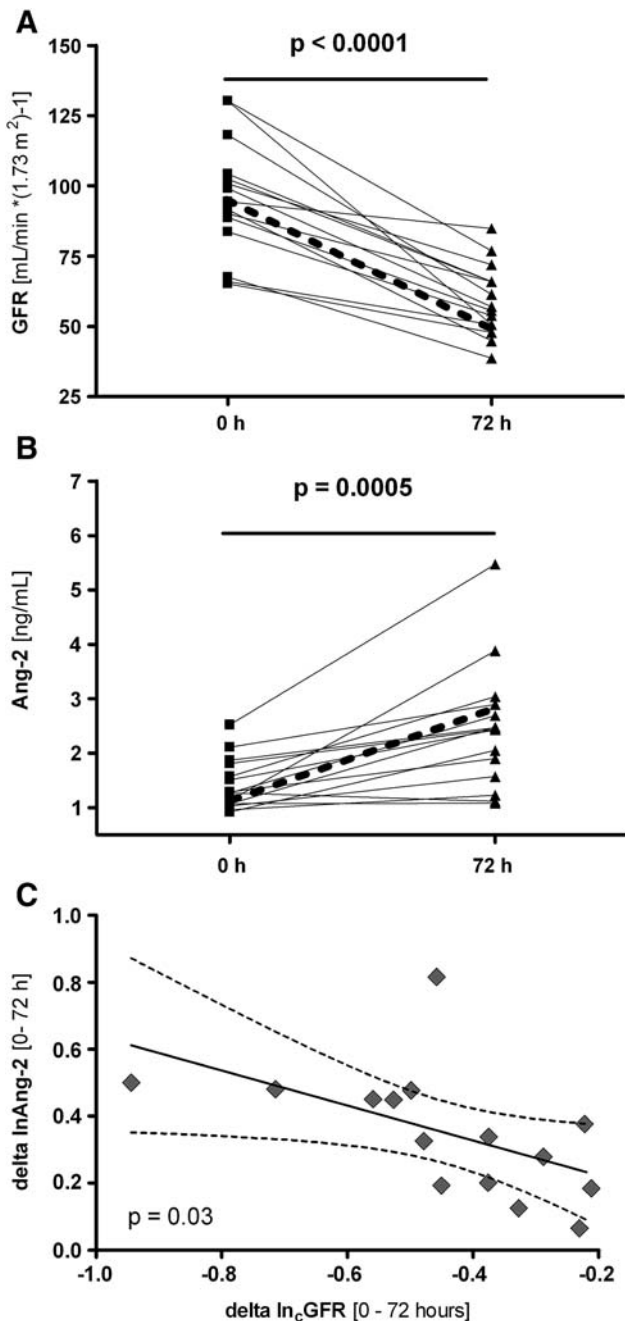
isons. The longitudinal data of kidney donors (i.e. Ang-2 level and GFR) were compared by non-parametric Friedman's tests for paired variables. Correlations between variables were assessed by the Spearman rank correlation coefficient. To fulfil the assumptions needed for the analysis, a logarithmic (ln) transformation of Ang-2 was performed. A multiple linear regression analysis with stepwise backward elimination was performed to identify the predictors of Ang-2 (dependent variable). Statistical significance was accepted at a two-sided  $P$ -value of  $< 0.05$ . The analyses were performed using the SPSS package (SPSS Inc., Chicago, IL, USA), and figures were prepared using the GraphPad Prism software (GraphPad Prism Software Inc. San Diego, CA, USA).

## Results

The clinical and demographic data of the healthy controls and CKD patients are shown in Table 1. The circulating Ang-2 was significantly higher in all CKD patients compared to the healthy controls [1.21 (0.85–3.10) ng/mL vs. 0.77 (0.32–1.08) ng/mL, respectively,  $P < 0.0001$ ].

#### Ang-2 levels increase with progress of CKD

Firstly, we assessed Ang-2 levels according to CKD stages. The median Ang-2 levels steadily increased across the following groups: healthy controls: 0.77 (0.32–1.08) ng/mL, CKD 1: 0.83 (0.67–1.09) ng/mL, CKD 2: 0.93 (0.74–1.15) ng/mL, CKD 3: 1.13 (0.87–1.49) ng/mL, CKD 4: 1.75 (1.23–2.61) ng/mL, and CKD 5: 4.87 (3.22–7.59) ng/mL, respectively (non-parametric ANOVA  $P < 0.0001$ ). The Ang-2 levels in patients with low CKD stages (i.e. 1 and 2) were not statistically different from the Ang-2 levels in healthy controls ( $P = 0.559$ ). However, at higher stages (i.e. CKD 3–5), we found a significant increase in circulating Ang-2 compared to healthy controls (CKD 3:  $P = 0.08$ ; CKD 4:  $P = 0.002$ ; CKD 5:  $P < 0.0001$ , Table 1 and Figure 1). According to dialysis modality, the patients in CKD stage 5 on maintenance haemodialysis showed the highest Ang-2 levels [HD: 5.64 (4.39–9.99) ng/mL; peritoneal dialysis (PD): 2.88 (2.49–3.78) ng/mL,  $P = 0.002$ , Figure 1]. Consistently, we detected a strong negative correlation between the  $iGFR$  and the circulating Ang-2 levels ( $r = -0.509$ ,  $P = 0.0004$ , Figure 2) in pa-



**Fig. 3.** Longitudinal course of (A) the cystatin C-estimated glomerular filtration rate (cGFR) before (0 h) and 72 h after unilateral nephrectomy, i.e. kidney donation, and (B) circulating Ang-2 levels (mean  $\Delta$  GFR:  $-35.6 \pm 18.6$  mL/min,  $P \leq 0.0001$ , mean  $\Delta$  Ang-2:  $\pm 1.02 \pm 0.88$  ng/mL,  $P = 0.0005$ ). Dotted lines represent median values. (C) Scatter plot showing the correlation of logarithmic (ln)  $\Delta$  Ang-2 level with the corresponding cystatin C-estimated GFR (ln  $\Delta$  cGFR) (Pearson's correlation:  $r = -0.54$ ,  $P = 0.03$ ). Dotted lines represent 95% confidence intervals.

tients with CKD stage 1–4 ( $n = 44$ ). Serum creatinine correlated similarly with Ang-2 ( $r = 0.506$ ,  $P < 0.0001$ ). Ang-1 levels were not significantly different between the CKD groups and did not correlate with the iGFR and ADMA (data not shown).

In addition to our findings in CKD patients, we investigated the effect of a sudden loss of GFR on the circulating

Ang-2 levels in subjects after the unilateral nephrectomy. We detected a decrease in mean cGFR ( $\Delta$  GFR:  $-35.6 \pm 18.6$  mL/min,  $P \leq 0.0001$ , Figure 3A) which was accompanied by an increase in circulating Ang-2 ( $\Delta$  Ang-2:  $\pm 1.02 \pm 0.88$  ng/mL,  $P = 0.0005$ , Figure 3B) 72 h after kidney donation. Consistently, a close inverse correlation between the mean changes (0–72 h) in Ang-2 and cGFR ( $r = -0.54$ ,  $P = 0.03$ , Figure 3C) was found.

#### Correlation of Ang-2 with classical and new cardiovascular risk factors

Next, we performed the bivariate correlation analyses for Ang-2 and several classical CV risk factors in CKD patients (1–4). Indeed, we detected significant results for the correlation with homocysteine ( $r = 0.365$ ,  $P = 0.015$ ) and serum phosphate ( $r = 0.53$ ,  $P < 0.0001$ ); both known to increase with loss of renal function [34]. All the other tested classical CV risk factors including patients' age ( $r = 0.149$ ,  $P = 0.335$ ), iPTH ( $r = 0.192$ ,  $P = 0.211$ ), BP ( $r = 0.04$ ,  $p = 0.79$ ), serum calcium ( $r = -0.28$ ,  $P = 0.064$ ), cholesterol ( $r = -0.234$ ,  $P = 0.13$ ) and triglycerides ( $r = -0.03$ ,  $P = 0.87$ ) were not correlated with serum Ang-2 level. Furthermore, there was no detectable influence of erythropoiesis-stimulating agents and/or vitamin D treatment on circulating Ang-2 levels.

According to our hypothesis, we further investigated the possible relation to ADMA, a major contributor to reduce NO bioavailability and thus potential trigger of WPB exocytosis. ADMA correlated significantly with circulating Ang-2 in stage 1–4 CKD patients ( $r = 0.35$ ,  $p = 0.01$ ). Interestingly, the association between ADMA and Ang-2 was even stronger when CKD stages 2–4 were analysed separately ( $r = 0.51$ ,  $P = 0.006$ ;  $n = 29$ ). In these subjects, ADMA remained a significant predictor of Ang-2 after adjusting for cGFR, serum phosphate and homocysteine in a multiple linear regression model ( $\beta = 0.372$ ,  $P = 0.034$ ).

## Discussion

To the best of our knowledge, this is the first study investigating the role of renal function on circulating Ang-2 level and linking Ang-2 to ADMA. We are able to show that: (i) Ang-2 levels steadily increase with the progression of CKD. (ii) There is a significant inverse correlation between Ang-2 and cGFR in CKD stages 1–4. (iii) Ang-2 correlates with several cardiovascular risk factors such as homocysteine and phosphate, as well as ADMA. (iv) The sudden loss of GFR after the unilateral nephrectomy in apparently healthy subjects is related to an increase in circulating Ang-2.

Previous clinical studies as well as experimental data from others indicate a pivotal role of Ang-2-driven endothelial activation in the pathogenesis of atherosclerosis. However, it is unknown if the kidney is indeed an important player in regulating circulating Ang-2 level rather than that the Ang-2 elevation just reflects prevalent vascular burden. Therefore, none of the participants had a known medical history of underlying PAD and/or CHD. Conse-

quently, the Ang-2 levels of the present study population were generally lower than in our previous cohort of stage 5 CKD patients with multiple comorbidities. Interestingly, the Ang-2 elevation first became evident in subjects with a GFR of <60 mL/min/1.73 m<sup>2</sup>.

To further study the role of intact renal function on the extent of circulating Ang-2, we included a second cohort consisting of 15 healthy kidney donors. Their Ang-2 levels increased shortly after nephrectomy and correlated with the decline in GFR.

These findings from loss of (kidney) function are well in line with our previous results in a gain of function model where elevated Ang-2 levels of dialysis patients normalized after a successful kidney transplantation [7].

There are three theoretical possibilities for how the Ang-2 homeostasis could be influenced by the kidney:

#### *First, reduced excretion of Ang-2 by the kidney*

Under denaturing conditions, purified recombinant Ang-2 protein exhibits predominant single bands of a molecular mass of ~62 kDa. Furthermore, *in vivo* Ang-2 exists mainly as a multimeric protein; thus, its glomerular excretion is rather unlikely. Ang-2 is neither detectable in urine from apparently healthy subjects (unpublished data) nor cleared by dialysis [32,35]. These observations argue against glomerular filtration or tubular secretion as physiologic routes of Ang-2 clearance from the circulation.

#### *Second, Ang-2 release by the impaired kidney*

The kidney endothelium itself has been identified as a rich source of Ang-2, so that chronic organ impairment might directly result in increased Ang-2 release from the kidney [26,29]. Although such a scenario is more likely to occur in acute kidney injury (AKI), it would not explain why Ang-2 increased in this study after the unilateral nephrectomy without inducing kidney disease in the remaining 'healthy' kidney.

#### *Third, CKD-related indirect release of systemic endothelial Ang-2*

CKD and the associated uraemia might trigger the release of Ang-2 from distant systemic endothelium via circulating 'uraemic toxins'.

It is conceivable to assume that the elevated Ang-2 levels in CKD patients might reflect excess WPB exocytosis as a consequence of decreased NO bioavailability in the presence of high ADMA levels. Indeed, we detected a weak albeit significant association of ADMA and Ang-2 in our CKD cohort. However, further *in vivo* and *in vitro* studies are needed to dissect the interaction between NO bioavailability and Ang-2 release.

Our study, which is hypothesis generating in nature, has several limitations. The major uncertainty we have to face is that we have no proof that circulating Ang-2 is biologically active in CKD patients. Presuming that this is the case, we still do not know the biological implication of Ang-2 changes in the range observed in our patients. Aside from this major limitation, there are several methodologi-

cal limitations of the study. The CKD population although well characterized, e.g. by inulin clearance is rather small. Furthermore, we limited the patients to two well-defined renal diseases that should not, in theory at least, cause changes in Ang-2 levels *per se*. The kidney donors have only been followed for 72 h; hence, we do not know if long-term changes in GFR will be accompanied by persistent changes in Ang-2 levels.

In summary, the current study provides a significant evidence that Ang-2 levels increase when increase in parallel to the deterioration of renal function. Since high Ang-2 concentrations enhance endothelial responsiveness towards various cytokines and growth factors, Ang-2 might act as an inflammatory sensitizer leading to vascular micro-inflammation and all its consequences in CKD. Future pre-clinical studies have to clarify the role of the kidney in Ang-2 homeostasis and might ultimately pave the road to therapeutic interventions alleviating the burden of CKD-associated atherosclerotic disease.

*Conflict of interest statement.* None declared.

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