Angiopoietin-2 levels predict mortality in CKD patients

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Abstract

Background. The pathophysiology of aggravated atherosclerosis in chronic kidney disease (CKD) is still incompletely understood. However, there is an increasing focus on non-traditional risk factors, including endothelial dysfunction. Angiopoietin-2 (Ang-2) impairs endothelial function by inhibiting the binding of Angiopoietin-1 (Ang-1) to their shared receptor Tie2 and is increased in diabetes, hypertension, coronary heart disease and CKD. Furthermore, Ang-2 levels are associated with the prevalent vascular burden of CKD patients. Thus, we aimed to investigate its impact on outcome in CKD, the population most likely to die of cardiovascular events.

Methods. We prospectively studied 128 CKD patients [43 CKD Stage 4, 85 CKD Stage 5 (57 haemodialysis, 28 peritoneal dialysis)] over a follow-up period of 4 years. Biochemical and clinical parameters, including objective scoring of vascular calcification (VC) by computed tomography (CT) and arterial stiffness by applanation tonometry (including radial-dorsalis pedis pulse wave velocity (PWVrd)) were recorded. Baseline Ang-1 [enzyme-linked immunosorbent assay (ELISA)], Ang-2 [immunoluminometric assay (ILMA)] and soluble Tie2 (sTie2) (ELISA) levels were measured in this group as well as in 20 healthy controls.

Results. Ang-2 values were significantly higher in CKD patients than in controls (2.01 ± 0.94 versus 1.00 ± 0.47 ng/mL, P < 0.0001). Furthermore, Ang-2 was significantly higher in dialysis than in Stage 4 CKD patients and correlated with markers of vascular disease [cholesterol, hsCRP, osteoprotegerin (OPG)]. However, elevated Ang-2 was not associated with the degree of VC or with arterial stiffness. Cox-regression analysis detected Ang-2 as an independent predictor of mortality in both unadjusted [hazard ratio (HR) 1.15; P = 0.002] and models adjusted for age and VC (HR 1.14; P = 0.003).

Conclusions. Ang-2 levels are associated with systemic markers/mediators of micro-inflammation in CKD patients. Furthermore, elevated Ang-2 levels are strong predictors of long-term mortality, independent of conduit arterial stiffness or VC.

Keywords: angiopoietin; atherosclerosis; CKD; dialysis; mortality

Introduction

The overall prevalence of treated end-stage renal disease (ESRD) in 2007 among all registries reporting to the European Renal Association-European Dialysis Transplantation Association Registry was 662 per million population [1]. The prevalence of chronic kidney disease (CKD) continues to grow at a significantly higher rate than the world population [2, 3] and accelerates cardiovascular (CV) burden immensely. Furthermore, patients suffering from less severe degrees of renal insufficiency, i.e. Stages 1–4 CKD are even more likely to die of cardiovascular disease (CVD) than develop overt kidney failure [4]. Of note, the pathogenesis of atherosclerosis-associated CVD in patients with CKD is different from that in the general population. Some new CV risk factors are more powerful indicating CV disease or endothelial dysfunction in CKD [e.g. osteoprotegerin (OPG), asymmetric dimethylarginine (ADMA), neuropeptide Y, visfatin] [5–9].

The angiopoietin (Ang)/Tie ligand—receptor system tightly controls the endothelial phenotype during angiogenesis and vascular inflammation in a unique and non-redundant fashion [10, 11]. The Tie2 receptor is almost exclusively expressed on endothelial cells (ECs). Its soluble form (sTie2) results from proteolytic cleavage of the full-length, membrane-bound receptor [12] and sTie2 has been implicated in the pathogenesis of CVD [13]. In mature vasculature, constitutive Ang-1-driven Tie2 phosphorylation probably represents a control pathway to maintain vascular quiescence by anti-apoptotic (Phosphatidylinositol 3 kinase/Akt signalling) and anti-inflammatory (Nuclear factor ‘kappa-light-chain-enhancer’ of activated B-cells...
signalling) properties, thus protecting the endothelium from excessive activation by cytokines and growth factors [11]. The rapid release of Ang-2 from ECs [14] upon activation of the endothelium (e.g. by thrombin, histamine or hypoxia) disrupts the protective, constitutive Ang-1/Tie2 signalling by preventing Ang-1 from binding to the receptor [11, 15]. Consequently, the loss of Tie2 signalling dramatically destabilizes the endothelium [16]. A long-term elevation in circulating Ang-2 could result in permanent endothelial micro-inflammation.

Consistently, increased levels of Ang-2 have been found in a variety of diseases known for their common characteristic of endothelial dysfunction/micro-inflammation (diabetes mellitus [17], cardiac allograft arteriosclerosis [18], acute coronary syndrome [13], systemic lupus erythematosus [19] and sepsis [20, 21]). We have recently reported that circulating Ang-2 is also markedly elevated in ESRD patients and that Ang-2 is closely associated with the prevalence of atherosclerosis [22].

However, our previous studies lack an objective quantification of prevalent vascular calcification (VC) as a possible end-result of Ang-2 driven micro-inflammation as well as an evaluation of patients’ outcome. Hypothesizing that Ang-2 levels might actively modulate atherosclerotic processes and VC thereby predicting mortality, we aimed to investigate: (i) the relationship between the Ang/Tie2 axis with both prevalent VC and arterial stiffness. (ii) The impact of the Ang/Tie2 system on mortality in a CKD population at high risk of death from CV events.

Material and methods

Patients

We studied 128 subjects [43 CKD Stage 4, 85 CKD Stage 5, 57 haemodialysis (HD), 28 peritoneal dialysis (PD)] recruited from Derby City General Hospital. Twenty apparently healthy volunteers without history of CKD or CV disease served as controls. The exclusion criteria for the CKD study population were previous transplantation, limb amputation or acute infection. CKD Stage 4 patients were defined by the four-variable Modification of Diet in Renal Disease (MDRD) study group reduces the risk of a CKD-independent Ang-2 elevation in those subjects. Exclusion criteria for the CKD study population were previous transplantation, limb amputation or acute infection. CKD Stage 4 patients were defined by the four-variable Modification of Diet in Renal Disease estimated glomerular filtration rate (eGFR) between 15 and 29 mL/min/1.73 m² averaged >6 months time-period prior to the visit date. Dialysis modal- ity was subject to patient choice; no patient had switched modality during the study. All dialysis patients were established for at least 6 months. PD patients were all treated with bicarbonate-lactate-buffered fluid (Physio- neal®; Baxter, Thetford, UK). Nine patients used automated PD, 19 patients used continuous ambulatory PD (three to five exchanges per day). HD patients received three sessions of at least 4 h/week using Hospal Integra (Mirandola, Italy) monitors, low-flux polysulphone dialysers (1.5–2.0 m², LOPS 15–20B; Braun Medical Ltd, Sheffield, UK) and bicarbonate-based HD with dialysate containing 1.25 mEq/L calcium and 134 mmol/L sodium in all sessions. Average Kt/Vurea was 1.2 ± 0.4 in HD (daily) and 2.4 ± 0.5 in PD patients (weekly). Standard dietary advice and support was provided to all subjects as standard policy. Daily sodium intake was limited to 100 mmol, daily protein 1.2 g/kg for at least 1-year prior to study. Appropriate ethical approval was granted by the South Derbyshire Local Research Ethics Committee and all patients provided written informed consent.

Data collection

Age, gender, CV co-morbidity, medication, height, weight, tobacco and alcohol use were recorded for all patients. CV co-morbidity was defined as any previous description of ischaemic heart disease, heart failure, cerebral vascular disease or peripheral vascular disease recorded in the patient’s medical notes. Biochemical parameters [haemoglobin, serum phosphate, serum corrected calcium, albumin ( bromocresol purple method) and cholesterol] were time-averaged over the previous 6 months. High sensitivity C-reactive protein (hsCRP) and OPG were assayed after inclusion of a patient by enzyme-linked immunosorbent assay (ELISA) (DRG Diagnostics, Marburg, Germany; Immunodiagnostic Systems, Boldon, UK).

Survival follow-up

Patient mortality was assessed 4 years after baseline using the computerized patient management system of the dialysis centre. We also investigated the underlying cause of death. Data for this analysis were only available from patients who died within the first 3 years after study inclusion.

Quantification of circulating Ang-1 and Ang-2 and sTie2

Blood samples were drawn on non-dialysis days in order to minimize alterations by the dialysis procedure itself. Plasma Ang-1 was measured by in-house ELISA and plasma Ang-2 by in-house immunoluminometric assay (ILMA) as described previously by our group [23]. In our hands, the assays had detection limits of 0.12 ng/mL (Ang-1) and 0.2 ng/mL (Ang-2). Inter-assay and intra-assay imprecision was 5.8% and 3.7% for Ang-1 and was 5.4% and 5.2% for Ang-2, respectively. Serum levels of sTie2 were measured by ELISA methodology (R&D Systems, Wiesbaden-Nordenstadt, Germany) according to the manufacturer’s instructions. All assays were performed in duplicate by investigators blinded to patients’ characteristics and outcome.

Quantification of VC

VC was quantified by peripheral multi-slice spiral computed tomography (CT) as previously described [24]. All studies were performed using GE Medical Systems LightSpeed16® multi-slice spiral CT scanner. Images were acquired without contrast when the patient was supine. A standardized section of the superficial femoral artery, 2 cm above the bifurcation level, 5 cm in length was imaged in 2.5 mm slices; care was taken to ensure that none of the slices overlapped. This allowed accurate reproducible quantification of calcium load in this section of artery. Calcification was considered to be present if an area denser than 130 Hounsfield units (HU) was ≥1 mm². The investigator scored each of the 20 slices individually, using GE Medical Systems® Advantage Workstation software. Thereby, each plaque score was generated as the product of the area and density. This method has previously been described in detail by Agatston et al. [25]. The investigator was blinded to the patients’ Ang and sTie2 levels.

Quantification of arterial stiffness

Arterial stiffness was assessed using pulse wave analysis (PWA) and pulse wave velocity (PWV) using a SphygmoCor® (AtCor Medical Pty Ltd, Sydney, Australia) system. Three blood pressure recordings were taken using an automated UA-767 oscillometric device. A 3-lead electrocardiograph (ECG) was attached to the subject and the surface distance between pulse points was measured. PWA was performed at the radial pulse. PWV was measured utilizing ECG-gated planimetry technique between both the carotid and radial pulses (PWVcr) and the radial and dorsalis pedis pulses (PWVrd). A single validated observer undertook all measurements on non-dialysis days.

Statistical analysis

Group data are presented as median (25–75 percentile) or as mean ± SD. All data were tested for normality. Categorical data was compared using Chi-square test, continuous data using paired or unpaired Student’s t-test, Mann–Whitney or Wilcoxon test and correlations using Pearson or Spearman tests as appropriate. The association of circulating Ang-2 with outcome was evaluated in adjusted Cox’s proportional hazards regression models. Selection of variables to be included in the multivariate models was done a priori by determining probable confounders based on differences in baseline characteristics between patients with different Ang-2 levels and based on theoretical considerations. The distribution of the time-to-event variables was estimated using the Kaplan–Meier method with log-rank testing. Analysis was performed using SPSS v12.0.1 (SPSS Inc, Chicago, IL, USA).

Results

Baseline Ang demographics among CKD subgroups

Patients from all three groups (CKD Stage 4, HD and PD) were well matched for general clinical and biochemical characteristics (Table 1).
## Table 1. Patients’ characteristics of CKD subjects

<table>
<thead>
<tr>
<th></th>
<th>CKD 4</th>
<th>PD</th>
<th>HD</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number (n)</td>
<td>43</td>
<td>28</td>
<td>57</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>64 ± 24</td>
<td>62 ± 14</td>
<td>65 ± 23</td>
<td>ns</td>
</tr>
<tr>
<td>Gender (male) (%)</td>
<td>60</td>
<td>61</td>
<td>72</td>
<td>ns</td>
</tr>
<tr>
<td>Diabetes mellitus (%)</td>
<td>23</td>
<td>36</td>
<td>26</td>
<td>ns</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>27 ± 8</td>
<td>26 ± 6</td>
<td>26 ± 7</td>
<td>ns</td>
</tr>
<tr>
<td>Dialysis vintage (months)</td>
<td>n/a</td>
<td>33 ± 23</td>
<td>37 ± 35</td>
<td>ns</td>
</tr>
<tr>
<td>CV co-morbidity (%)</td>
<td>26</td>
<td>43</td>
<td>18</td>
<td>0.049</td>
</tr>
<tr>
<td>Smokers (%)</td>
<td>14</td>
<td>14</td>
<td>11</td>
<td>ns</td>
</tr>
<tr>
<td>Use of ACEi (%)</td>
<td>35</td>
<td>43</td>
<td>18</td>
<td>0.028</td>
</tr>
<tr>
<td>Use of β-blocker (%)</td>
<td>44</td>
<td>11</td>
<td>26</td>
<td>0.006</td>
</tr>
<tr>
<td>Use of statins (%)</td>
<td>37</td>
<td>50</td>
<td>32</td>
<td>ns</td>
</tr>
<tr>
<td>Use of erythropoietin (%)</td>
<td>20</td>
<td>68</td>
<td>93</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>5.1 ± 0.9</td>
<td>4.5 ± 1</td>
<td>4.2 ± 1</td>
<td>0.013 &lt;0.001 ns</td>
</tr>
<tr>
<td>Serum corrected calcium (mmol/L)</td>
<td>2.38 ± 0.1</td>
<td>2.51 ± 0.1</td>
<td>2.46 ± 0.1</td>
<td>ns</td>
</tr>
<tr>
<td>Serum phosphate (mmol/L)</td>
<td>1.47 ± 0.3</td>
<td>1.59 ± 0.3</td>
<td>1.69 ± 0.4</td>
<td>ns</td>
</tr>
<tr>
<td>PTH (pg/mL)</td>
<td>179 ± 167</td>
<td>293 ± 219</td>
<td>292 ± 250</td>
<td>ns</td>
</tr>
<tr>
<td>Albumin (g/L)</td>
<td>34 ± 3</td>
<td>28 ± 6</td>
<td>34 ± 3</td>
<td>ns</td>
</tr>
<tr>
<td>eGFR (MDRD four-variable) (mL/min/1.73 m²)</td>
<td>16.2 ± 7.5</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>Kt/V (single-session HD, weekly PD)</td>
<td>n/a</td>
<td>2.39 ± 0.48</td>
<td>1.20 ± 0.4</td>
<td>n/a</td>
</tr>
<tr>
<td>VC (U)</td>
<td>2 ± 198</td>
<td>26 ± 487</td>
<td>121 ± 623</td>
<td>0.045 0.11 ns</td>
</tr>
<tr>
<td>Survival (%)</td>
<td>85%</td>
<td>61%</td>
<td>72%</td>
<td>ns</td>
</tr>
</tbody>
</table>

*BMI body mass index, ACEi angiotensin converting enzyme inhibitor, PTH parathyroid hormone, CV co-morbidity was defined as any previous description of ischaemic heart disease, heart failure, cerebral vascular disease or peripheral vascular disease recorded in the patient’s medical notes, ns not significant, n/a not available.

### Baseline parameters associated with Ang-1, Ang-2 and sTie2

At baseline, there was no significant difference in Ang-1 levels between the CKD groups, but CKD patients [CKD 4: 6.8 (4.55–10.63) ng/mL; PD: 6.7 (4.7–9.5) ng/mL; HD: 5.7 (3.75–7.2) ng/mL] had generally higher levels than healthy controls [2.5 (1.6–5.7) ng/mL, Figure 1A]. Similarly, circulating Ang-2 was higher in the CKD population (grouped together) than in healthy controls [2.0 (1.67–2.6) versus 0.99 (0.89–1.37) ng/mL, P < 0.0001]. However, Ang-2 levels were also higher in CKD 4 when compared to healthy controls [1.81 (1.46–2.34) versus 0.99 (0.89–1.37) ng/mL; P < 0.0001]. Furthermore, Ang-2 levels were higher in dialysis patients than in CKD 4 patients [2.08 (1.79–2.73) versus 1.81 (1.46–2.34) ng/mL; P = 0.01], but no difference was observed between dialysis modalities [HD 2.06 (1.76–2.74) ng/mL; PD 2.17 (1.84–2.64) ng/mL; P = 0.43, Figure 1B]. Similar relationships were found when we looked at the ratio between Ang-2/Ang-1, (Figure 1D). Levels of sTie2 steadily increased across the following groups: controls [1.26 (1.05–1.4) ng/mL], CKD 4 patients [2.32 (2.05–2.6) ng/mL], PD patients [2.96 (2.5–3.52) ng/mL] and HD patients [3.41 (3.0–4.2) ng/mL, Figure 1C].

Furthermore, Ang-1 and Ang-2 (r = 0.246; P = 0.048) as well as Ang-1 and sTie2 (r = 0.324; P = 0.001) were positively correlated with each other.
sTie2 levels correlated with albumin ($r = -0.303; P = 0.002$), total cholesterol ($r = -0.23; P = 0.020$), OPG ($r = 0.27; P = 0.006$), and hsCRP ($r = 0.28; P = 0.006$).

**Correlation with prevalent VC and arterial stiffness at baseline**

There was no association between the CT-assisted measurement of VC and Ang-1 or Ang-2. Similarly, arterial stiffness did not correlate with both growth factors. Even when the current cohort was investigated separately for CKD Stages 4 and 5, we could not detect any association with VC (data not shown). However, sTie2 correlated significantly with arterial stiffness (PWVrd, $r = 0.237; P = 0.028$) and VC ($r = 0.217; P = 0.031$).

**Mortality/outcome**

There were 38 overall-deaths (29.7%) over 1.495 days of follow-up recorded. CV events account for 47% of deaths, cancer for 8% and the rest were a mixture of sepsis and unknown causes. Interestingly, only circulating Ang-2 levels predicted mortality in both unadjusted [hazard ratio (HR) 1.15; $P = 0.002$] and Cox-regression models adjusted for various risk factors (Table 2). When visualized by Kaplan–Meier curves, mortality steadily increased among Ang-2 tertiles [Log-rank (Mantel–Cox) test for trend $P = 0.03$, Figure 2]. In an unadjusted model age (HR 1.054, $P < 0.001$), VC (HR 10.33, $P < 0.001$), male gender (HR 3.947, $P = 0.001$) and CV co-morbidity (HR 2.872, $P = 0.003$) also predicted mortality. Ang-1 and sTie2 levels were not predictive, either alone or in combination with Ang-2 (Supplementary Figure 1).

**Discussion**

To the best of our knowledge, this is the first prospective clinical study investigating the impact of circulating angiopoietins (Ang-1 and Ang-2) and sTie2 in predicting CKD patients’ outcome over an observation period of 4 years. We could show that: (i) Ang-2 levels were elevated in CKD Stages 4–5 patients. (ii) Baseline Ang-2 levels independently predict long-term mortality. (iii) However, there was
no association between the elevation of circulating Ang-2 with an objective score of VC or arterial stiffness.

The current study population was thoroughly chosen as a result of recent findings that highlighted the fact that Ang-2 elevation first becomes evident in subjects with a GFR of <60 mL/min/1.73 m² [26]. Furthermore, in CKD 4–5, the suspected mortality over a 4-year observation period was high enough to investigate the predictive impact of this new biomarker family. This discovery is entirely new and adds knowledge to our up-to-date understanding of the disease. It also might help physicians to estimate patients’ risks of death and thereby focussing on those patients. In times of dominating importance on guidelines-steered therapies, Ang-2’s potential in predicting outcome might help us identify those patients at very high risk in our every day care in dialysis units.

Several recent studies found an association between circulating Ang-2 and other markers as well as mediators of endothelial dysfunction. Therefore, we wanted to test here if the same is true for a population known for its somehow different pathogenesis of atherosclerotic disease. And indeed, even in our CKD patients, we found significant relationships between Ang-2 and established (cholesterol, albumin, hsCRP) as well as new (OPG) markers/mediators of vascular disease.

Our own group recently showed that Ang-2 levels are associated with prevalent atherosclerosis in CKD Stage 5 patients on maintenance dialysis. In spite of taking different vascular beds into account, the used scoring system was arbitrarily chosen and semi-quantitative in nature. Now, we attempted to add evidence to these preliminary results using a highly objective method of scoring VC by means of CT scans of the iliac arteries followed by a computer-assisted analysing system. Furthermore, we quantified arterial stiffness measured by PWV in all our patients and correlated the results with circulating levels of Angs and sTie2. Interestingly, we could not confirm our previous findings as an association between Ang-2 and VC in the present cohort was undetectable.

There are some theoretical explanations for this discrepancy. Firstly, the lack of an association might simply reflect a lower grade of underlying prevalent atherosclerosis in the present cohort due to the inclusion of Stage 4 CKD patients. Even when CKD Stage 5 was assessed separately, patients had much lower prevalent CV disease than in our own earlier studies. This theory is supported by the very weak relation between hsCRP and Ang-2 that points towards a lower grade of baseline micro-inflammation in this population. Secondly, in spite that most of the atherosclerotic plaques contain calcifications, not all calcification are located at sites of plaque deposition. In other words, not all calcified vessels represent atherosclerotic lesions.

Thirdly, the highly non-normal distribution of the calcification score might also contribute to the lack of an association between VC and Ang-2. Last but not least, VC was only assessed in the iliac arteries. Taking into account, the heterogeneity of the endothelium, one might speculate that other vascular beds (such as the coronary endothelium) might be more susceptible to alterations in the Ang/Tie axis. Theoretically, this could result in a better correlation between prevalent coronary heart disease and Ang-2 than when only correlated with peripheral arterial disease. This lack of association was unexpected. However, Ang-2 triggered micro-inflammatory response of the endothelium (as shown before [16]) could still be responsible for CV events even without resulting in manifest atherosclerotic VC.

PD patients had higher CV co-morbidities and higher VC compared to HD patients. This finding might be biased by our own clinical routine treating haemodynamically less stable patients that suffer from a high degree of CKD-associated CV-morbidities preferentially with PD.

The correlation reported here between sTie2 and VC as well as arterial stiffness might reflect the interplay between various players in the Ang/Tie ligand—receptor system. Soluble receptor forms have been found to be elevated as a result of shear stress-induced shedding of the membrane-bound receptor form. One might argue that shear stress could be a direct cause of VC and thereby explains our sTie2 finding.

Interestingly, we found slightly higher Ang-2 levels in those patients on statin therapy. This finding was unexpected, as we have recently shown in a prospective randomized multi-centre trial with ~300 hypertensive patients that statins did not have a direct influence on Ang-2 levels [27]. These different results might be based on the fundamental differences of the two studied populations (i.e. CKD was an exclusion criteria in the EUTOPIA trial).

The present study has several limitations. Although this CKD population is big enough to answer our question regarding patients’ outcome, it is still a rather small study population and the study has been performed in a single-centre setting. Circulating levels of Ang-2 in the present investigation were lower than those reported earlier in CKD patients, probably reflecting an overall lower prevalence of vascular disease. The fact that we cannot provide our patients’ coronary status represents an important limitation of the present study.

In summary, this prospective observational study provides significant evidence that increased Ang-2 levels in CKD Stage 4–5 patients are useful as a predictor of mortality, independent of conduit arterial stiffness or VC.
Conflict of interest statement

David is a scholar of the German Research Foundation (DFG) (DA 1209/1-1). Else-Kröner-Fresenius Foundation (P63/06/EKMS 06/03). Dr Sascha

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Conflict of interest statement. None declared.

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Supplementary data

Supplementary Figure 1 is available online at http://ndt.oxfordjournals.org/