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Circulating Angiopoietin-2 levels predict mortality in kidney transplant recipients: a 4-year prospective case–cohort study

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Summary

Angiopoietin 2 (Angpt2) impairs endothelial function by preventing angiopoietin 1 from binding to their common endothelial-specific receptor Tie2. Here, we examined whether circulating Angpt2 predicts outcome in kidney transplant recipients. For this case–cohort study, we selected 130 kidney transplant recipients who had died or returned to dialysis within the first 2 years of follow-up of our cohort study, as well as 130 age- and gender-matched kidney transplant recipients without an event (controls) from a total of 993 kidney transplant recipients. The total of 260 selected patients were followed in median 4 years. Serum Angpt2 at baseline was measured using an in-house immunoluminometric assay. Median Angpt2 concentrations were significantly higher in patients who died [median (interquartile range – IQR) 3.6 (2.8–5.9) ng/ml] as compared to patients who did not die during the study period [2.8 (2.1–4.1) ng/ml; $P < 0.001$]. Ln (natural log) Angpt2 levels correlated positively with C-reactive protein levels ($r = 0.315$, $P < 0.001$) and the Charlson Comorbidity Index ($r = 0.188$, $P = 0.002$) and were inversely associated with eGFR ($r = -0.301$, $P < 0.001$) hemoglobin ($r = -0.269$, $P < 0.001$), and serum albumin concentrations ($r = -0.382$, $P < 0.001$). On multivariate analyses, baseline Angpt2 levels independently predicted all-cause mortality (multivariable-adjusted hazard ratio associated with one natural log unit higher Angpt2 level: 1.70 (95% confidence interval: 1.10–2.61)). In our analysis, circulating Angpt2 was an independent predictor of all-cause mortality in stable, prevalent kidney transplant recipients.

Introduction

Kidney transplantation is the treatment of choice for patients with chronic kidney disease (CKD)5D, that is, patients undergoing chronic hemodialysis treatment. Kidney transplantation is associated with better outcomes compared with maintenance dialysis therapy [1–4]. Patients after renal transplantation, however, still have a fourfold to fivefold higher mortality compared with the general population [5–8]. The leading cause of death after renal transplantation is cardiovascular disease (CVD) [9]. Traditional cardiovascular risk factors such as age, gender, and comorbidity have been reported to not fully predict mortality in this population [10–13]. Hence, reports about

the impact of nontraditional risk factors such as micro-inflammation, carotid artery intima–media thickness or endothelial dysfunction that highlighted the impact of the vascular endothelium in the complex pathophysiology of cardiovascular disease in kidney transplant patients have generated much interest from basic scientists and clinicians [14,15]. Recently, the angiopoietin (Angpt)/Tie2 ligand–receptor system presented as a promising biomarker and pathophysiologically important factor in CVD research [16–20].

The angiopoietin–Tie2 signaling tightly controls the endothelial phenotype during angiogenesis and also vascular inflammation in a unique and nonredundant fashion [21–23]. As circulating or matrix-bound molecules, angiopoietin-1 (Angpt1) and its antagonist angiopoietin-2 (Angpt2) bind to the extracellular domain of the tyrosine kinase receptor Tie2, which is almost exclusively expressed on endothelial cells. In the adult vasculature, a delicate balance of constitutive Angpt1 expression by vascular smooth-muscle cells (SMCs) and precursor pericytes, and low-level Tie2 phosphorylation seem to control and maintain vascular quiescence [24,25], thus protecting the endothelium from excessive activation by cytokines and growth factors [26]. Ang-1/Tie2 signaling can be easily suppressed by binding of Angpt2 to the common Tie2 receptor [21,27,28].

Angpt2 is expressed in endothelial cells, where it is stored in Weibel–Palade bodies (WPB) [29]. The rapid release of Angpt2 upon activation of the endothelium (e.g. by thrombin, histamine or hypoxia) disrupts the vessel-protective Angpt1/Tie2 signaling by preventing Angpt1 from binding to the receptor [22,30]. The loss of Tie2 signaling renders the endothelium responsive toward a variety of pro-inflammatory cytokines and growth factor [31]. The importance and nonredundancy of Ang-1/Tie2 signaling is illustrated by either Ang-1 $^{-/-}$ or Tie2 $^{-/-}$ knockout mice, which die *in utero* owing to severe vascular remodeling defects causing perturbed vascular integrity [16,18].

Increased circulating Angpt2 was found in a variety of diseases known for their common characteristic of vascular inflammation and endothelial activation such as diabetes mellitus [32], cardiac allograft arteriosclerosis [33], acute coronary syndrome [34], systemic lupus erythematosus [35], and sepsis [36–38]. Ang2 levels are significantly increased in patients with CHF when compared with healthy controls [39, 40]. Serum Ang2 correlates with impaired exercise capacity and reduced ventilatory capacity in patients with CHF [39,40]. It has recently been suggested that Ang2 is a marker of early cardiovascular disease in children with CKD [20]. We have earlier shown that circulating Angpt2 increases with the progression of chronic kidney disease (CKD) [41], correlates with the severity of coronary and peripheral vascular disease in patients on maintenance dialysis [42], and predicts mortality in patients with CKD stages 4–5 [43]. Although Angpt2 levels return toward normal after successful kidney transplantation [42], Angpt2 remains a putative cardiovascular risk factor in this population. In addition to these observational data, experimental studies demonstrated that manipulation of the angiopoietin–Tie system will lead to endothelial or glomerular damage or protection, depending on the direction of the changes [23] lending further support to the potential importance of this signaling system.

Based on the aforementioned data, we assessed the association of Angpt2 and all-cause mortality both in patients with functioning graft and after returning to dialysis (primary outcome). Furthermore, we also analyzed whether Angpt2 predicts "death with functioning graft" (secondary outcome). We hypothesized that circulating Angpt2 levels predict all-cause mortality in kidney transplant recipients. We tested this hypothesis in a prospective case–cohort study of prevalent kidney transplant recipients.

Patients and methods

Patient population and data collection

All prevalent kidney transplant recipients 18 years of age or older (n = 1214) who were followed at a single transplant outpatient clinic in the Department of Transplantation and Surgery at the Semmelweis University, Budapest on December 31, 2006 were invited to participate in a prospective cohort study [Malnutrition-Inflammation in Transplant – Hungary Study (MINIT-HU Study)] [44–48]. Exclusion criteria were acute rejection within the last 4 weeks, current hospitalization, transplantation in the previous 3 months, acute infection, or bleeding. Baseline assessments were conducted between February 2007 and August 2007.

The sample of the subcohort for the purpose of the current case–cohort study was selected after 2-year outcome data have become available in 2009 (Fig. 1). We selected 130 patients who had died (60 patients) or had returned to dialysis (70 patients) during the first 2 years of follow-up. One hundred and thirty age- and gender-matched controls were selected from the remainder of the cohort (Fig. 1). This case–control subsample was then considered as a single subcohort of the MINIT-HU cohort. We followed all patients enrolled in this subcohort for about an additional 2 years after patient selection, and we collected outcome data during this extended follow-up period until July 1, 2011. In this analysis, all-cause mortality (both with a functioning graft and after returning to dialysis) was the principal outcome for the entire subcohort. Thus, the current analysis was based on 49 months of follow-up between the baseline assessment in 2007 and 2011 (Fig. 1).

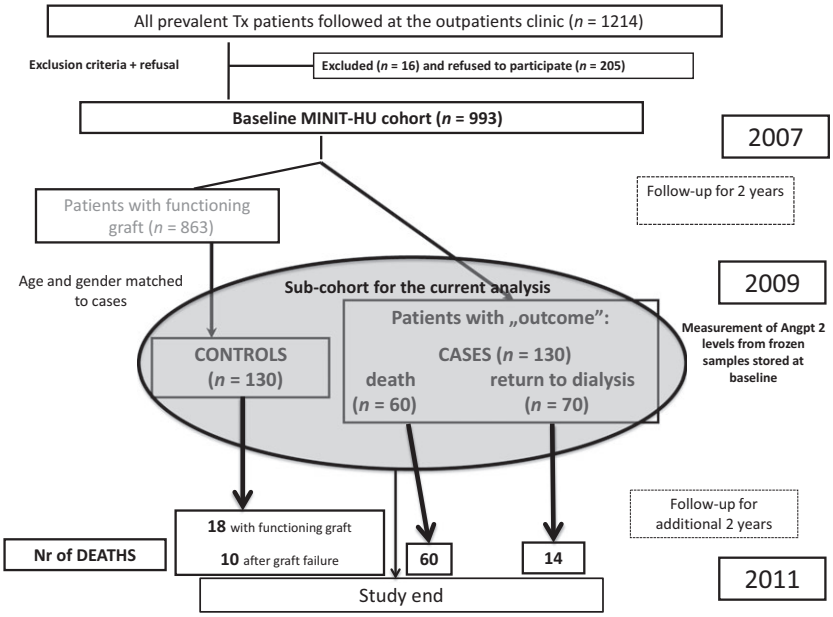

Figure 1 Selection of the patients.

Demographic data and details of medical history were collected at enrollment, when information about age, gender, and menopause status, etiology of CKD, transplantation-related data including immunosuppressant medication use, weight, height, abdominal circumference, and comorbidities, including the modified Charlson Comorbidity Index (CCI) [49], were obtained. Estimated glomerular filtration rate (eGFR) was calculated using Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation [50].

Comorbidity

One of the most commonly used and validated indexes for kidney transplant populations is the Charlson Comorbidity Index (CCI) [51]. We used the modified CCI [49], which is a weighted scoring system based on the presence or absence of each of 17 variables. As one of the variables is the presence of moderate to severe renal disease, the minimum score for all patients with established stage 5 CKD is 2. Thus, in patients with established stage 5 CKD, the score ranges from 2 to a possible maximum of 33. Previously, the CCI has been shown to predict survival in kidney transplant patients [49]. The presence of hypertension (based on physician report), which is not included in the Charlson Comorbidity Index, was also assessed. Blood pressure was measured during outpatient visits (three times after ten-minute rest), tabulating the average of the three measurements.

Transplantation-related data and donor characteristics

Transplant related data extracted from the medical records included the following information: current medications (including current immunosuppressive treatment), transplant "vintage," that is, time elapsed since the time of the transplantation, previous time on dialysis, type of allograft (4% of allografts came from living donors), history of acute rejection after transplantation, HLA mismatch, panel-reactive antibodies titer (PRA), cold ischemia time, donor age and gender (62% of donors were male and mean donor age was 44 ± 15 years), and history of delayed graft function. Delayed graft function was defined as the need for at least one hemodialysis session in the first week after transplantation [52].

Immunosuppressive therapy

Standard maintenance immunosuppressive therapy generally consisted of prednisolone, either cyclosporine A microemulsion formulation (Neoral) (CsA) or tacrolimus, combined with mycophenolate mofetil (MMF) or azathioprine or sirolimus or everolimus.

Follow-up

Patients were followed for 49.4 months [median, (interquartile range – IQR): 49.4, (23.9–51.8) months]. The primary outcome variable was all-cause mortality (includes all deaths with functioning graft or after return to dialysis).

Secondary outcome measure was death with functioning graft. The time of death and of re-initiation of maintenance dialysis were ascertained from the hospital database. Deaths were also validated by cross-referencing with data from the Hungarian Central Office of Administrative and Electronic Public Service. The clinical and research activities being reported are consistent with the Principles of the Declaration of Istanbul as outlined in the 'Declaration of Istanbul on Organ Trafficking and Transplant Tourism.

Laboratory data

Laboratory measurements were obtained at the time of each prespecified visit. In addition to Angpt2, the following laboratory parameters were tabulated for this study: hemoglobin (Hb), C-reactive protein (CRP), serum creatinine, blood urea nitrogen (BUN), and serum albumin.

Blood sampling and quantification of circulating Angpt2

Baseline blood samples were collected at study entry. Circulating serum Angpt2 concentrations were measured by in-house immunoluminometric assay (ILMA) as previously reported by our group in detail [53]. The coefficient of variation was <6%. The sensitivity threshold was 0.2 ng/ml. All assays were performed in duplicate by a single investigator blinded to patients' characteristics and outcome.

Statistical analysis

Statistical analyses were carried out using STATA 11.1 (StataCorp LP, College Station, TX, USA) and SAS, version 9.1 (SAS Institute Inc., Cary, NC, USA) software. Data were summarized using proportions, means (±standard deviation, SD), or medians (interquartile range, IQR) as appropriate. Categorical variables were analyzed with chi-square test, and continuous variables were compared using Student's *t*-test or the Mann–Whitney *U*-test, Kruskal–Wallis H test, or ANOVA as appropriate. In all statistics, two-sided tests were used and the results were considered statistically significant if P was <0.05. The association between the circulating Angpt2 level and all-cause mortality was assessed using Cox proportional regression analysis and Kaplan–Meier plots with log-rank test. Plots of log [−log (survival rate)] against log (survival time) were used to check the proportionality assumption. As death with functioning graft and graft loss are competing events, the association of circulating Angpt2 level with death with functioning graft was assessed by means of semiparametric competing-risks regression analyses [54]. Only baseline variables were used in the semiparametric competing-risks regression analysis. We also assessed this association using left-truncated analysis as sensitivity analysis.

The variables entered in the multivariable-adjusted models were selected on theoretical considerations; we included predictors in the models which were known to be associated with graft loss or mortality based on external evidence and clinical experience, and which were available in our database. Age, gender, eGFR, Charlson Comorbidity Index, blood hemoglobin, serum albumin, serum phosphate, and average of systolic blood pressure were entered as baseline data.

Circulating Angpt2 level was analyzed both as a categorical variable based on median of serum Angpt2 level (≤3.2 ng/ml vs. >3.2 ng/ml) and as a continuous variable. Serum Angpt2 level and natural logarithm-transformed serum Angpt2 level was analyzed as a continuous variable, because of skewed distribution of circulating Angpt2 level. Nonlinear associations were assessed using fractional polynomials and restricted cubic splines. Additionally, we tested the nonlinearity by adding the quadratic term of circulating Angpt2 level to the models already containing the linear term. We also tested for nonlinearity in all of our models using the "mvrs" STATA command to examine whether any of the covariates have a nonlinear association with all-cause mortality. Variance inflation factors (VIF) were used to assess collinearity between independent variables. Less than 1% of our data were missing for any variables.

Results

Association of Angpt2 levels with baseline characteristics

Baseline characteristics of the study sample of 260 patients are shown in Tables 1–2. The mean age was 54 ± 13 years, the mean eGFR was 43 ± 21 ml/min/1.73 m^2, 58% were male and 22% were diabetic (Table 1). The median and interquartile range (IQR) of circulating angiopoietin-2 level was 3.2 (2.3–4.9) ng/ml.

Circulating Angpt2 level showed a moderate negative correlation with serum albumin ($R = -0.382$, $P < 0.001$), blood hemoglobin ($R = -0.269$, $P < 0.001$) and eGFR ($R = -0.301$, $P < 0.001$), and moderate positive correlation with ln CRP level ($R = 0.315$, $P < 0.001$) and Charlson Comorbidity Index ($R = 0.188$, $P < 0.001$), as shown in Fig. 2. In addition, serum Angpt2 levels were significantly correlated with serum asymmetric dimethylarginine (ADMA) and serum symmetric dimethylarginine (SDMA) levels ($r = 0.196$, $P = 0.0015$; $r = 0.339$, $P < 0.001$, respectively).

Association of Angpt2 levels with clinical outcomes

During a median follow-up of 49 months, 102 patients died and none were lost to follow-up. The distribution of all deaths in this cohort is as follows (Fig. 1): 60 patients died within 2 years of study entry with functioning graft (initial

Table 1. Patients' baseline characteristics divided by cases and controls.

	All patients (n = 260)	Cases (n = 130)	Controls (n = 130)	P-value*
Angpt2 (ng/ml) [median (IQR)]	3.2 (2.3–4.9)	3.9 (2.5–5.9)	2.9 (2.1–3.7)	<0.001
Age (year) (mean ± SD)	54 ± 13	54 ± 12	53 ± 13	0.511
Gender (male) (%)	58	61	56	0.450
Time since last transplant (month) [median (IQR)]	88 (52–123)	95 (59–124)	80 (38–117)	0.036
Previous time on dialysis (month) [median (IQR)]	20 (9–39)	18 (9–36)	22 (9–48)	0.363
eGFR (MDRD) (ml/min/1.73 m^2) (mean ± SD)	42 ± 21	32 ± 18	53 ± 18	<0.001
Charlson Comorbidity Index [median (IQR)]	2 (2–4)	3 (2–5)	2 (2–3)	<0.001
Presence of hypertension (%)	95	97	94	0.237
Average systolic blood pressure (mmHg) (mean ± SD)	146 ± 20	151 ± 21	141 ± 18	<0.001
Average diastolic blood pressure (mmHg) (mean ± SD)	85 ± 13	86 ± 14	83 ± 11	0.054
Presence of diabetes (%)	22	24	20	0.454
Smoking (%)	20	19	21	0.756
Hgb (g/l) (mean ± SD)	129 ± 19	122 ± 20	136 ± 15	<0.001
Serum albumin (g/l) (mean ± SD)	39 ± 5	37 ± 5	40 ± 4	<0.001
CRP (mg/l) [median (IQR)]	3.7 (1.6–7.3)	3.6 (1.4–8.1)	3.8 (1.6–7.0)	0.887
Primary cause of ESRD (%)				
Chronic GN	23	22	25	0.365
Chronic TIN	11	12	9	
PKD	21	19	23	
Diabetic nephropathy	3	2	4	
Hypertensive nephropathy	9	12	5	
Others or unknown	33	33	34	
Steroid therapy (%)	87	91	82	0.046
Cyclosporine A therapy (%)	50	51	49	0.804
Tacrolimus therapy (%)	37	36	38	0.700
Mycophenolate mofetil therapy (%)	73	71	75	0.482
Azathioprine therapy (%)	5	5	5	0.999
Sirolimus therapy (%)	10	9	10	0.833
Everolimus therapy (%)	3	4	2	0.309
Cold ischemic time (min.) (mean ± SD)	1253 ± 366	1266 ± 357	1241 ± 375	0.587
History of delayed graft function (%)	28	27	29	0.656
Panel-reactive antibody (%) [mean ± SD; (median; min–max)]	3.2 ± 10.9 (0; 0–80)	3.9 ± 11.1 (0; 0–60)	2.5 ± 10.8 (0; 0–80)	0.018
History of acute rejection (%)	40	44	37	0.234
HLA mismatches (%)				
0	1	2	0	0.807
1	4	4	4	
2	19	20	16	
3	52	50	54	
4	18	17	20	
5	5	6	5	
6	1	1	1	

Angpt2, serum angiopoietin 2; ESRD, end-stage renal disease; eGFR, estimated GFR; Hgb, hemoglobin; CRP, C-reactive protein; PKD; polycystic kidney disease; GN, glomerulonephritis; TIN, tubulo-interstitialis nephritis.
*Comparing Angpt2 ≤ 3.2 ng/ml and Angpt2 > 3.2 ng/ml groups.

"caseness" defined by death). A total of 18 additional patients died with functioning graft (initial "controls"). A total of 24 patients died after returning to dialysis, 14 patients who started dialysis within the first 2 years ("caseness by graft loss") and 10 patients, initially "controls", who returned to dialysis during the third-fourth year of follow-up, and died subsequently. Median baseline Angpt2 concentrations were significantly higher in patients who died [median (interquartile range – IQR): 3.6 (2.8–5.9) ng/ml] as compared to patients who did not die during the study period [2.8 (2.1–4.1) ng/ml; $P < 0.001$]. The crude mortality rate was 121/1000 patient-years [95% confidence interval (CI): 100–147]. The unadjusted mortality rate was significantly higher among patients with circulating Angpt2 levels above versus below the median [crude mortality rate: 181 (95% CI: 142–231) vs. 76 (95% CI: 55–105) per 1000 patient-years; $P < 0.001$] (Fig. 3). Moreover, higher circulating Angpt2 levels were also associated with increased

Table 2. Baseline characteristics of the cohort divide by median of angiopoietin 2 level.

	Angpt2 ≤ 3.2 ng/ml (*n* = 135)	Angpt2 > 3.2 ng/ml (*n* = 125)	*P*-value*
Angpt2 (ng/ml) [median (IQR)]	2.3 (1.9–2.8)	5.0 (3.8–6.2)	<0.001
Age (year) (mean ± SD)	53 ± 13	55 ± 12	0.285
Gender (male) (%)	61	56	0.438
Time since last transplant (month) [median (IQR)]	76 (44–119)	98 (61–131)	0.010
Previous time on dialysis (month) [median (IQR)]	18 (9–36)	22 (10–42)	0.519
eGFR (CKD-EPI) (ml/min/1.73 m^2) (mean ± SD)	48 ± 19	39 ± 23	<0.001
Charlson Comorbidity Index [median (IQR)]	2 (2–3)	2 (2–4)	0.004
Presence of hypertension (%)	95	96	0.649
Average systolic blood pressure (mmHg) (mean ± SD)	144 ± 19	148 ± 22	0.069
Average diastolic blood pressure (mmHg) (mean ± SD)	85 ± 12	85 ± 14	0.957
Presence of diabetes (%)	21	23	0.632
Smoking (%)	17	23	0.215
Hgb (g/l) (mean ± SD)	133 ± 18	125 ± 19	0.001
Serum albumin (g/l) (mean ± SD)	40 ± 4	37 ± 5	<0.001
CRP (mg/l) [median (IQR)]	2.8 (1.3–5.3)	4.2 (2.1–9.0)	0.001
Steroid therapy (%)	90	83	0.129
Cyclosporine A therapy (%)	48	52	0.535
Tacrolimus therapy (%)	36	38	0.726
Mycophenolate mofetil therapy (%)	76	71	0.427
Azathioprine therapy (%)	4	5	0.891
Sirolimus therapy (%)	12	7	0.204
Everolimus therapy (%)	4	3	0.824
Cold ischemic time (min.) (mean ± SD)	1234 ± 377	1274 ± 354	0.393
History of delayed graft function (%)	27	29	0.663
Panel-reactive antibody (%) [mean ± SD; (median; min.–max.)]	2 ± 7.6 (0; 0–55)	4.5 ± 13.6 (0; 0–80)	0.024
History of acute rejection (%)	36	45	0.178
HLA mismatches (%)			
0	1	1	0.714
1	4	4	
2	18	18	
3	51	54	
4	20	16	
5	4	7	
6	2	0	

Angpt2, serum angiopoietin 2; ESRD, end-stage renal disease; eGFR, estimated GFR; Hgb, hemoglobin; CRP, C-reactive protein.
*Comparing Angpt2 ≤ 3.2 ng/ml and Angpt2 > 3.2 ng/ml groups.

deaths with functioning graft, as shown by the adjusted cumulative incidence curves (Figure S1).

In multivariable Cox proportional regression analyses, the circulating Angpt2 level was identified as a significant predictor of all-cause mortality ($HR_{1\ ng/ml\ increase}$ = 1.10; 95% CI: 1.00–1.21, P = 0.045) (Table 3). Patients with serum Angpt2 levels above the median (3.2 ng/ml) had 86% higher risk of all-cause mortality compared to patients with circulating Angpt2 levels < 3.2 ng/ml (HR: 1.86; 95% CI: 1.19–2.90, P = 0.006) (Table 3). Additionally, we found qualitatively similar results using a left-truncated analysis to account for the prevalent cohort nature of our sample (Table S1). In an additional model, which included serum CRP instead of serum albumin (to avoid potential collinearity), Angpt2 was still significantly associated with total mortality [HR: 1.12 (95% CI 1.02–1.23, P = 0.021)]. The association between the risk of death with functioning graft and higher circulating Angpt2 levels trended in the same direction ($HR_{1\ ng/ml\ increase}$ = 1.11; 95% CI: 0.98–1.26, P = 0.094) when analyzing the data with semiparametric competing-risks regression analysis to account for the competing nature of the outcomes of graft loss and risk of death with a functioning graft (Table S2).

Serum Angpt2 level was significantly associated with all-cause mortality when modeled as a continuous variable and using fractional polynomials and cubic splines (Fig. 4). The hazard ratios were monotonously up-going until 6 ng/ml at which concentration it seemed to reach a plateau. The quadratic term of circulating Angpt2 level was not significant in any of these models. To characterize the accuracy of serum Angpt2 in predicting all-cause mortality, we calculated C statistic and sensitivity/specificity for the median

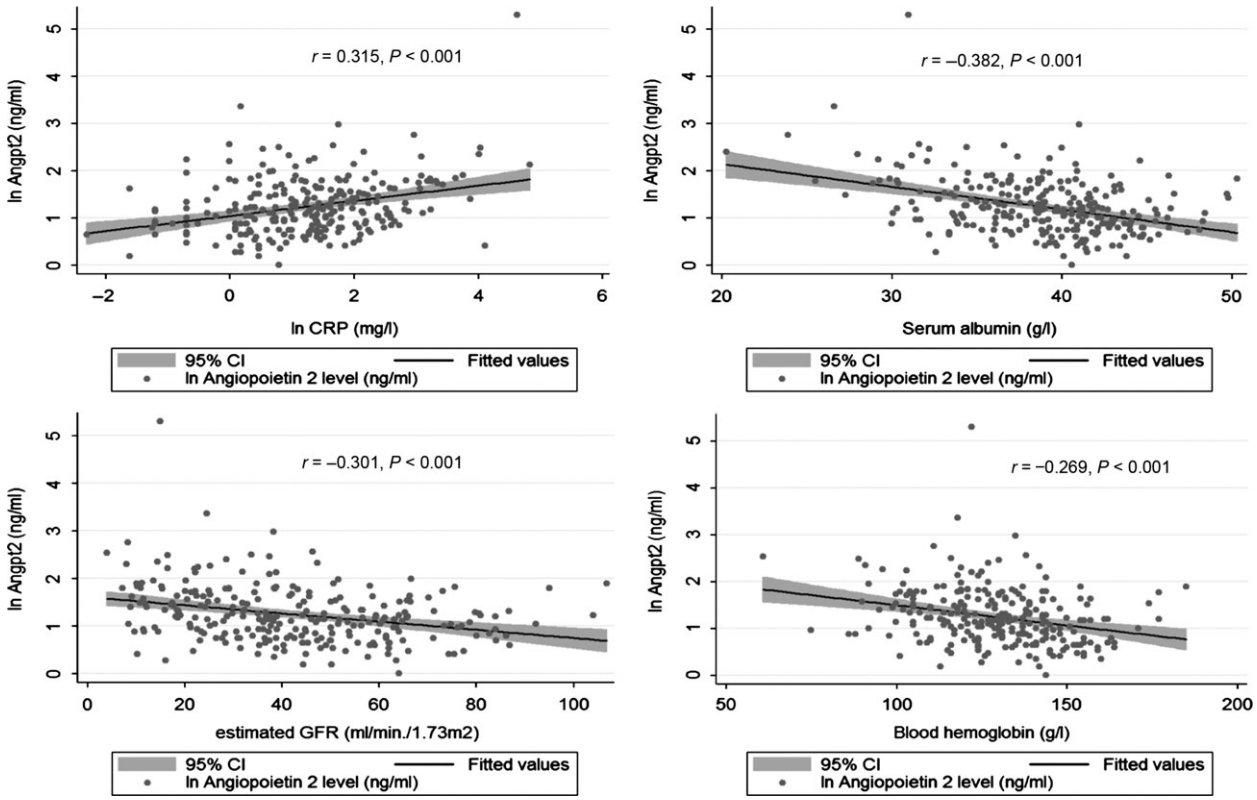

Figure 2 Correlation between serum angiopoietin 2 level and markers of inflammation (C-reactive protein, serum albumin), residual renal function (estimated GFR), and blood hemoglobin.

Angpt2 level (Table 2). The discriminating accuracy of serum Angpt2 level was similar to that of serum albumin (not shown).

In our dataset, serum Angpt2 levels were associated with graft loss in un-adjusted analysis, but this association was not significant after adjusting for potentially impor-

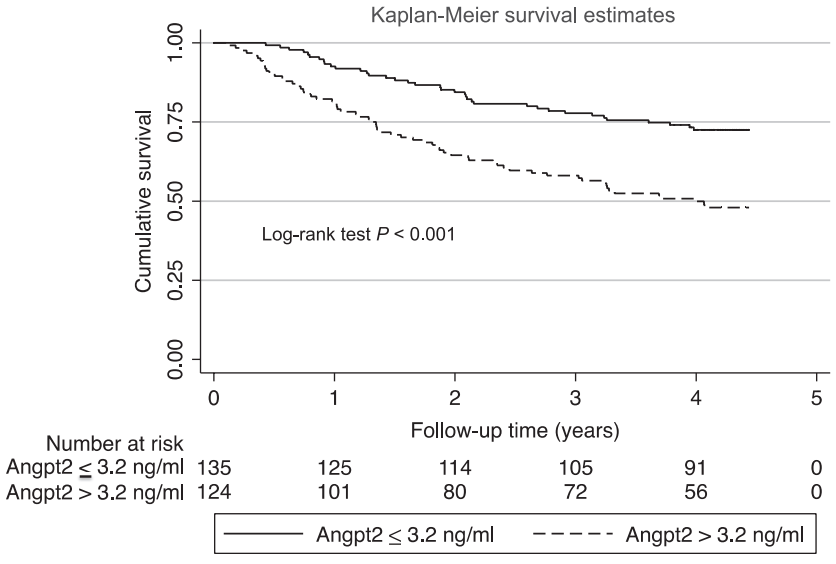

Figure 3 Kaplan–Meier analysis of all-cause mortality for 260 kidney transplant patients according to serum angiopoietin 2 level.

Table 3. Association of serum angiopoietin 2 level with all-cause mortality in Cox proportional regression analyses.

	Univariate model for mortality			Multivariable model for mortality*		
	Number of death = 102			Number of death = 99		
	HR	95% CI	*P*	HR	95% CI	*P*
Angpt2 (+1 ng/ml increase)	1.19	1.10–1.28	<0.001	1.10	1.00–1.21	0.045
Ln Angpt2 (+1 Ln ng/ml increase)	2.35	1.62–3.39	<0.001	1.70	1.10–2.61	0.016
Angpt2 (≤3.2 ng/ml vs. >3.2 ng/ml)	2.34	1.57–3.52	<0.001	1.86	1.19–2.90	0.006

Angpt2, serum angiopoietin 2; HR, hazard ratio; CI, confidence interval.
*Adjusted for: age, gender, eGFR, Charlson Comorbidity Index, blood hemoglobin, serum albumin, serum phosphate, and average of systolic blood pressure.

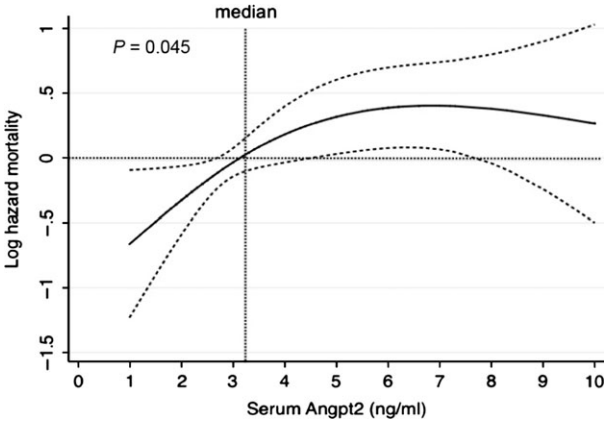

Figure 4 Association of serum angiopoietin 2 level with all-cause mortality in Cox proportional hazard models. Models were adjusted for age, gender, eGFR, Charlson Comorbidity Index, blood hemoglobin, serum albumin, serum phosphate, and average of systolic blood pressure.

tant covariables, such as age and baseline eGFR (not shown).

Discussion

The salient findings of this nested case–cohort study is that circulating Angpt2 levels independently predict all-cause mortality in a prevalent cohort of 260 stable kidney transplant recipients. Patients with increased serum Angpt2 (≥3.2 ng/ml) levels had 86% higher risk of all-cause mortality compared to patients with circulating Angpt2 levels < 3.2 ng/ml. The association of the risk of death with functioning graft and higher circulating Angpt2 levels was similar, but not significant due to lower event numbers. To our knowledge, this study is the first to demonstrate the association between Angpt2 levels and all-cause mortality in kidney transplant patients. Although there was an association between serum Angpt2 levels and graft loss in unadjusted analysis, this association lost significance after adjusting for age and baseline eGFR (no shown).

Previous clinical studies as well as experimental data from others indicate a pivotal role of Angpt2-driven endothelial activation in the pathogenesis of vascular inflammation and atherosclerosis. For example, Angpt2 expression is upregulated in (unstable) atherosclerotic plaques and correlates with matrix metalloproteinase 2 (MMP-2) activity and plaque microvascular density [55]. Angpt2 expression is tightly controlled. Low Angpt2 mRNA expression in the quiescent vasculature is strongly upregulated following stimulation by cytokines, growth factors, and environmental factors (hypoxia, high glucose levels, and oxidative stress) [17,56]. Hence, Angpt2 functions as a dynamic autocrine-negative regulator of the quiescent resting endothelium and as a key destabilizing signal involved in initiating angiogenic remodeling [57]. Mice deficient in Angpt2 hardly elicit vascular inflammation in experimental peritonitis and are protected from leukocyte transmigration in the dorsal skinfold chamber model *in vivo* [26]. Similarly, rodents receiving adenoviral constructs encoding an engineered Angpt1 variant are largely protected from target organ damage in diabetes [58] and hypertension [59]. Accordingly, it has repeatedly been suggested that the angiopoietin–Tie signaling system is a potential therapeutic target in malignancy and in cardiovascular disease [23,60].

In patients with diabetes, plasma Angpt2 (but not Angpt1) levels are increased, correlate with indices of endothelial dysfunction (e.g. carotid artery intima–media thickness and microalbuminuria) [32], and even decline after intensive CV risk management [61]. Patel *et al.* [62] could show that plasma levels of Angpt2 (but not Angpt1 or the soluble Tie-2 receptor) were consistently associated with CVD outcomes during a 5-year follow-up period among a subgroup of 251 patients with hypertension from Anglo-Scandinavian Cardiac Outcomes Trial (ASCOT) study. Of note, median Angpt2 levels in these patients were markedly lower [1.3 (1.9–3.4)] than levels detected in the current study [3.2 (2.3–4.9)]. Consistent with this notion, all-cause mortality was ~13-fold higher in the

current study (9.3/1000 vs. 121/1000 patient-years). It is thus conceivable to assume that transplantation-related factors, such as graft function and micro-inflammation, increase Angpt2 expression and that higher Angpt2 levels impact on outcome in our patients. Indeed, Angpt2 levels were inversely related to eGFR in the current study. This finding is in line with our previous data showing that Angpt2 levels (but not Angpt1) increase with the progression of renal function in patients with CKD, even in the absence of overt CVD. Interestingly, increased Angpt2 in these patients first became evident in subjects with CKD 3 (i.e. GFR <60 ml/min/1.73 m^2). Of note, the average eGFR in the current study was 43 ml/min/1.73 m^2. However, Angpt2 is neither cleared nor secreted by the kidney [41]. Although the pathophysiologic link (if any) between increased Angpt2 serum levels versus the progression of CKD is not clear, there is some evidence that increased Angpt2 might reflect excess WPB exocytosis as a consequence of decreased nitric oxide bioavailability [41].

As high Angpt2 concentrations enhance endothelial responsiveness toward various cytokines and growth factors *in vitro*, Angpt2 might act as an inflammatory sensitizer leading to vascular micro-inflammation in patients with CKD. Increased inflammation itself and several potentially related factors (such as anemia and endothelial dysfunction) are associated with mortality in kidney transplant patients [46,48,63,64]. Consistent with this notion, we found a highly statistically significant association between increased Angpt2 levels and indices of micro-inflammation, such as low serum albumin, low blood hemoglobin, and increased CRP levels. In addition, Angpt2 levels were also significantly correlated with markers of endothelial dysfunction, such as serum ADMA and SDMA.

Importantly, we found a robust and consistent association between serum Angpt2 versus all-cause mortality even after adjustment for age, gender, eGFR, Charlson Comorbidity Index, blood hemoglobin, serum albumin, serum phosphate, and average of systolic blood pressure. Moreover, the association between circulating Angpt2 and all-cause mortality was confirmed in several sets of sensitivity analyses, which emphasizes the robust association between high circulating Angpt2 versus outcome in kidney transplant patients. These results extend previous data on the association between serum Angpt2 levels and mortality in various acute [65,66] and chronic conditions [43,67], and even in the general population [68].

Several limitations of our study deserve discussion. First, patients were enrolled from a single center. This cohort was a prevalent cohort, which is subject to incidence–prevalence bias. Moreover, patients who were not participating in the study were likely different from the participants, which also could result in bias. Another limitation is the relatively small size of the cohort and relatively small number of events. We did not have information about the cause of death in this cohort, although detailed analysis of cause of death in these transplant recipients would have substantially improved the usefulness of the reported findings. We believe, however, that it is likely that serum Angpt2 levels are mainly associated with cardiovascular disease and cardiovascular death in this patient population, as well. This, however, will have to be tested in further studies. Therefore, our results should not be generalized without further considerations. Second, by its very nature, our observational study cannot prove causal associations between predictors and outcomes. Third, indices of vascular inflammation and subclinical atherosclerosis, such as carotid intima–media thickness, were not assessed in this study. Fourth, potential confounders, such as proteinuria, hepatitis B virus, hepatitis C virus and cytomegalovirus infection, and data for surgical complications were not accounted for in our analysis. Finally, we did not measure circulating Angpt1 and VEGF levels, because falsely high Angpt1 serum levels may result from unavoidable *in vitro* platelet degranulation [53]. The same is probably true for vascular endothelial growth factor [69]. However, from a pathophysiologic perspective, it might be highly desirable to evaluate Angpt2 in the context Angpt1 and VEGF.

In summary, our case–cohort study demonstrated that higher circulating Angpt2 is independently associated with increased all-cause mortality risk in stable kidney transplant patients. We believe that our results will stimulate mechanistically targeted hypothesis generation about mortality in kidney transplant recipients, because this signaling system has repeatedly been considered as potential therapeutic target [23,26,60].

Authorship

MZM: designed, organized, and coordinated the study, managed data entry, contributed to data analysis and interpretation of data and wrote the manuscript. PK: contributed to analyzing and interpretation of data and writing the manuscript. JTK: contributed to analyzing and interpretation of data and writing the manuscript. MS: contributed to analyzing and interpretation of data and writing the manuscript. MEC: contributed to analyzing and interpretation of data and writing the manuscript. AU: contributed to analyzing and interpretation of data and writing the manuscript. CPK: contributed to analyzing and interpretation of data and writing the manuscript. IM: designed, organized, and coordinated the study, managed data entry, contributed to data analysis and interpretation of data, and wrote the manuscript.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1. Association of serum angiopoietin 2 level with all-cause mortality in Cox proportional regression analyses–left-truncated analysis.

Table S2. Association of serum angiopoietin 2 level with death with functioning graft in semiparametric competing-risks regression analyses.

Table S3. C statistic, sensitivity, specificity, positive predictive value and negative predictive values for serum Angpt2 to predict all-cause mortality in our cohort.

Figure S1. Multivariable-adjusted cumulative incidence curve of death with functioning graft in patients with different serum levels of angiopoietin 2.

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