Angiopoietins in renal replacement therapy

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CHAPTER 4

Angiopoietin-2 associates with graft failure and mortality in renal transplant recipients

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ABSTRACT

Background

Angiopoietin-1 (Ang1) and angiopoietin-2 (Ang2) are involved in stabilizing vascular endothelium and may play a role in mortality and graft failure in renal transplant recipients (RTR). Early identification of RTR at risk could allow management, possibly via anti-Ang2 therapy. We aimed to investigate the association of Ang1 and Ang2 with graft failure and mortality in a prospective cohort of RTR. Elevated Ang2 levels have been demonstrated in sepsis which has pathophysiological similarities to the deceased brain dead donor. Therefore we also separately studied Ang2 associations in RTR transplanted with deceased donor kidneys.

Methods

Plasma Ang1 and Ang2 levels were measured in 552 RTR and 86 living kidney donors (LKD).

Results

Ang1 was higher in RTR than in LKD (p=0.002), while Ang2 was similar. For Ang1, no association with heart rate, Nt-pro-BNP or hsCRP was observed. Similar multivariate analysis demonstrated associations with Ang2 (all p<0.001). In deceased donor-RTR, Ang2 levels were higher compared to living donor-RTR. After adjustment for potential confounders, Ang2 levels were associated with graft failure (HR 3.64, 95%CI 1.17-11.28, p=0.03) and mortality (HR 1.53, 95%CI 1.03-2.27, p=0.04) after deceased donation.

Conclusions

Studies investigating the mechanism of the Ang/Tie2-system in renal transplantation are needed to provide more insight on cause and effect.

INTRODUCTION

The angiopoietin/Tie2 ligand-receptor system is involved in stabilizing the vascular endothelium and has been proposed as a potential therapeutic target in various conditions[1-3]. Angiopoietins are ligands that bind to the tyrosine kinase receptor Tie2, which is almost exclusively expressed by endothelial and hemopoietic stem cells[4]. Binding of Ang1 to the Tie-receptor leads to stabilization of the endothelium. In contrast, Ang2 destabilizes the blood vessels and enhances vascular leakage by priming the endothelial cells to respond to cytokines[5]. Ang1 is produced and immediately released at a constant rate by precursor platelets, pericytes and vascular smooth-muscle cells (SMCs) while Ang2 is stored in Weibel Palade bodies (WPB)[4,6,7]. The content of these endothelial-specific storage granules is rapidly released upon endothelial activation in response to, among others, thrombin, histamin and superoxide. Release of Ang2 leads to inflammation, coagulation and angiogenesis[4,5,8]. In the adult vasculature, a delicate balance of constitutive Ang1 expression and low-level Tie2 phosphorylation controls and maintains vascular quiescence, thus protecting the endothelium from excessive activation[9,10]. Ang1 and Ang2 therefore not only play important roles in the autocrine regulation of vascular stability and permeability, but also in the inflammatory balance.

Angiopoietin-2 (Ang2) is currently being evaluated as a promising biomarker in the field of acute pancreatitis[11]. Elevated Ang2 plasma levels have also been associated with a range of conditions such as myocardial infarction, trauma and sepsis[12,13]. Moreover, circulating Ang2 increases with the progression of chronic kidney disease (CKD), is predictive of mortality in CKD patients and correlates with severity of vascular disease in patients on dialysis[6,14,15]. Anti-Ang2 therapies have been studied in several preclinical models and anti-Ang2 phase III clinical trials that have been performed to date provided promising results[16-20].

For patients with end stage renal disease, renal transplantation has become the treatment of choice. However, graft failure remains an important problem and mortality rates remain high in RTR compared to the general population[21-27]. The origin of this high rate of mortality is multi-factorial with a high prevalence of a multitude of synergistically acting risk factors including morbidity, micro-inflammation and impaired graft function[24,26,28-31]. The involvement of endothelial dysfunction interacting with proteinuria in increasing the risk for mortality in RTR highlights the importance of vascular endothelium[32-34].

Ang2 release is triggered in human experimental endotoxemia and in sepsis elevated Ang2 is associated with increased mortality[35-37]. The known endotoxemia in DBD donors together with the pathophysiological similarities between sepsis and brain death makes the Ang/Tie2-system an interesting interventional target in DBD donors. Especially since endothelial activation has been shown to be a key factor in organs derived from DBD donors[38,39].

We aimed to investigate Ang1 and Ang2 levels among RTR and healthy controls. Additionally, we investigated associations of Ang1 and Ang2 with clinical parameters in RTR. We moreover hypothesized that Ang1 and Ang2 are associated with the occurrence of graft failure and mortality in stable, outpatient RTR. Because of the suggested role of Ang2 in endothelial activation in deceased donor kidneys, secondary analyses were performed in recipients of a kidney derived from deceased donors.

MATERIALS AND METHODS

Study populations

For the current analysis, we used plasma of a prospective observational single-center cohort study in which all RTR (≥ 18years) with a functioning graft, who visited our outpatient clinic between 2008 and 2010, were invited to participate[40]. A total of 707 out of 817 eligible RTR (87%) signed written informed consent. Plasma Ang1 and Ang2 levels were measured in 552 RTR (78.1%). As a healthy control group, we included 86 subjects after they signed written informed consent, which were evaluated and approved for living kidney donation in our center. None had a history of kidney disease or diabetes mellitus. Hypertension, if present, was treated with a maximum of three antihypertensive drugs. The Institutional Review Board approved the study protocol (METc 2008/186), which was in adherence to the Declaration of Helsinki.

Urine and plasma parameters

All participants were carefully instructed to collect a 24-hour urine sample according to a strict protocol at the day prior to their visit to the outpatient clinic. Urine was collected with chlorhexidin was added as antiseptic agent. Blood was drawn in the morning after completion of the 24h urine collection. For the control group, the 24h urine collection was performed before donor nephrectomy. Plasma Ang1 and Ang2 levels were measured via enzyme-linked immunosorbent assay (ELISA) Duosets (R&D Systems, Minneapolis, USA).

Plasma and urinary concentrations of albumin, HbA1c, hsCRP, Nt-pro-BNP and total protein levels were measured using a Roche Modular chemistry analyzer (Roche Diagnostics, USA).

Renal function was assessed by estimating Glomerular Filtration Rate according to the Chronic Kidney Disease Epidemiology Collaboration (eGFR). Serum creatinine was determined using an enzymatic essay on a Roche Modular analyzer.

Clinical parameters

All measurements were performed during a morning visit to the outpatient clinic after an 8-12hr overnight fasting period. Blood pressure (mmHg) of RTR and living

donors was measured according to a strict protocol as previously described[41]. Participants were in half-sitting position while systolic blood pressure (SBP), diastolic blood pressure (DBP), mean arterial pressure (MAP) and heart rate were measured with a semi-automatic device (Dinamapâ 1846, Critikon, Tampa, FL, USA). Measurements were performed every minute for fifteen minutes and the last three values were averaged. Information on participants' health status, medical history and medication use was obtained from patient records. Information about the renal transplantation was extracted from the local University Medical Center Groningen renal transplantation database. Body weight and height were measured with participants wearing indoor clothing without shoes. BMI was calculated as weight divided by height squared (kg/m^2) and Body Surface Area (BSA) was estimated applying the universally adopted formula of Dubois and Dubois[42].

Clinical endpoints

The primary endpoints of this study were mortality and death-censored graft failure defined as return to dialysis or re-transplantation. The continuous surveillance system of the outpatient program ensures up-to-date information on patient status and cause of death. General practitioners or referring nephrologists were contacted in case the status of a patient was unknown. Endpoints were recorded until the end of May 2013. There was no loss to follow-up.

Statistical analyses

Data are presented as mean ± SD (standard deviation), median (range) or [interquartile range] (IQR) and number (percentage) for normally, non-normally distributed data, and nominal data, respectively. Analyses were performed using SPSS version 22.0 (SPSS Inc., Chicago, IL, USA). Normality was tested with the Kolmogorov-Smirnov test. Skewed data were normalized for analyses by natural-logarithm transformation (LN) transformation. In all analyses, a two-sided *P*-value of less then 0.05 was considered to indicate statistical significance. Differences between RTR and healthy controls, and deceased donor- RTR vs. living donor-RTR, were tested with the t-test for independent samples or the Mann Whitney U test in case of continuous variables and the Chi-square test in case of dichotomous variables. Age and sex-adjusted associations of Ang1 or Ang2 levels with various clinical parameters were analyzed with linear regression analysis (model 1). Further adjustments were made for eGFR and transplantation vintage (model 2), use of antihypertensives (model 3) and additional adjustment for either Ang1 or Ang2 (model 4).

Regression coefficients are given as standardized betas. In prospective analyses, we investigated associations of plasma Ang1 and Ang2 with death-censored graft failure and all-cause mortality in RTR. Subsequently, associations of plasma Ang1 and Ang2 with graft failure and mortality were studied separately in RTR with a graft retrieved from a deceased or living donor. Kaplan-Meier survival curves

and logrank tests were performed between groups to asses the difference in death-censored graft survival or patient survival rates. We performed crude Cox regression analyses for the association with death-censored graft failure (model 1). Additionally analyses were adjusted for age, sex and donor type (model 2), cold ischemia time, number of transplantations, number of mismatches (model 3), and years since transplantation, albuminuria (mg/24h) and renal function (eGFR) (model 4). For the association with all-cause mortality, we first performed crude Cox regression analyses (model 1) and adjusted for age and sex (model 2), and additionally adjusted for diabetes and donor type (model 3), and additionally adjusted for years since transplantation and renal function (eGFR) (model 4). Possible interactions between confounders were tested.

RESULTS

RTR vs. controls

The characteristics of RTR and controls are shown in table 1. Baseline measurements in RTR were performed at a median of 5.4 [IQR 1.9-12.2] years after kidney transplantation. RTR and controls were similar with respect to age, sex, BMI and BSA. Plasma Ang1 concentration was higher in RTR; 1715 (range 134-9776) vs. 1207 (151-5638) in controls, p=0.002, but Ang2 concentration did not differ between both groups (607 (0.12-2467) in controls vs. 665 (0.96-9242) in RTR; p=0.14). As anticipated, serum creatinine and eGFR were significantly worse in RTR than in healthy subjects (both p<0.001). This was accompanied by higher blood pressure values in RTR than in controls (both p<0.001). Compared to controls, RTR had significantly higher levels of albumin excretion, glycosylated haemoglobin (HbA1c) and Nt-pro-BNP (all p<0.001). Of the 552 RTR, 355 recipients received a kidney from a deceased donor, 197 recipients received a kidney from a living donor. A comparison of RTR according to donor type is also presented in table 1. Plasma Ang1 concentration did not differ between these groups (p=0.96). Ang2 levels were higher among RTR of a deceased donor kidney (p=0.003). Deceased donor-RTR had significantly higher levels of Nt-pro-BNP compared to living donor-RTR (p<0.001).

Independent associations of Ang1 and Ang2 levels with clinical parameters in RTR

Age and sex-adjusted regression coefficients for the associations of plasma Ang1 and Ang2 with cardiovascular parameters are presented in table 2 (model 1). After adjustment for potential confounders (model 2), plasma Ang1 was significantly associated with DBP (β=0.07; p=0.04). Further adjustment for potential confounders did not change the significant associations of Ang2 with heart rate (β=0.12; p=0.001), Nt-pro-BNP (β=0.12; p<0.001) and hsCRP (β=0.17; p<0.001). The association of Ang2 with HbA1c was lost after adjustment for the use of antihypertensives (model 4).

Table 1. Baseline characteristics of 86 healthy controls and 552 renal transplant recipients at the day of their visit to the outpatient clinic

Characteristics	Healthy controls n=86	RTR n=552	p	RTR of DD n=352	RTR of LD n=200	p
Plasma Ang1 (pg/ml)	1207 [151-5638]	1715 [134-9776]	0.002	1738 [141-9601]	1656 [134-9776]	0.471
Plasma Ang2 (pg/ml)	607 [0.12-2467]	665 [0.96-9242]	0.141	721 [5-9242]	591 [1-6860]	0.003
Demographics						
Age (y)	43 ± 16	53 ± 13	0.417	55 ± 12	49 ± 13	<0.001
Male sex, n (%)	50	54.3	0.45	64	36	0.61
BMI (kg/m^2)	27 ± 3.5	27 ± 4.7	0.399	27 ± 5	26 ± 4	0.208
BSA (m^2)	1.97 ± 0.2	1.93 ± 0.2	0.178	1.94 ± 0.2	1.95 ± 0.2	0.320
Medication use						
Antihypertensive (%)	16.3	87.7	<0.001	90	85	0.094
Statins (%)	3.5	52.4	<0.001	54	51	0.736
Hemodynamic parameters						
SBP (mmHg)	125 ± 15	136 ± 18	<0.001	136 ± 18	136 ± 16	0.911
DBP (mmHg)	76 ± 10	82 ± 11	<0.001	82 ± 11	84 ± 11	0.005
MAP (mmHg)	97 ± 18	107 ± 15	<0.001	107 ± 15	108 ± 15	0.277
Heart rate (bpm)	66 ± 9	69 ± 12	0.068	68 ± 12	69 ± 11	0.026
Renal function parameters						
eGFR (ml/min)	90 ± 17	50 ± 18	<0.001	48 ± 19	51 ± 18	0.080
Serum creatinine (μmol/L)	73 ± 13	134 ± 51	<0.001	140 ± 60	135 ± 58	0.270
Urinary protein (gram/24h)	0.06 ± 0.1	0.36 ± 0.75	<0.001	0.45 ± 0.9	0.33 ± 0.7	0.010
Albumin excretion (mg/L)	3.3 ± 5.3	99 ± 257	<0.001	124 ± 292	107 ± 306	0.026
Serum parameters						
HbA1c (%)	5.5 ± 0.3	6 ± 0.8	<0.001	6 ± 0.8	6 ± 0.9	0.123
hsCRP (mg/L)	2.9 ± 7.6	4.3 ± 9.2	0.072	4.8 ± 9.4	3.8 ± 8.9	0.179
Nt-pro-BNP (ng/L)	56 ± 58	1018 ± 5414	<0.001	1458 ± 6398	382 ± 630	<0.001

Data are presented as mean ± SD, % or median [range]. Abbreviations: RTR: renal transplant recipients; BMI, body mass index; BSA: body surface area; SBP: systolic blood pressure; DBP: diastolic blood pressure; MAP: mean arterial pressure; eGFR: estimated Glomerular Filtration Rate according to the Chronic Kidney Disease Epidemiology Collaboration; HbA1c: glycosylated hemoglobuline; hsCRP: high-sensitive C-Reactive Protein; Nt-pro-BNP: N-terminal pro-Brain Natriuretic Peptide. P for difference was tested by the Mann-Whitney U test for continuous variables or Chi-square test for binary variables.

Table 2. Regression coefficients for the association of plasma Ang1 and Ang2 with clinical parameters in 552 renal transplant recipients

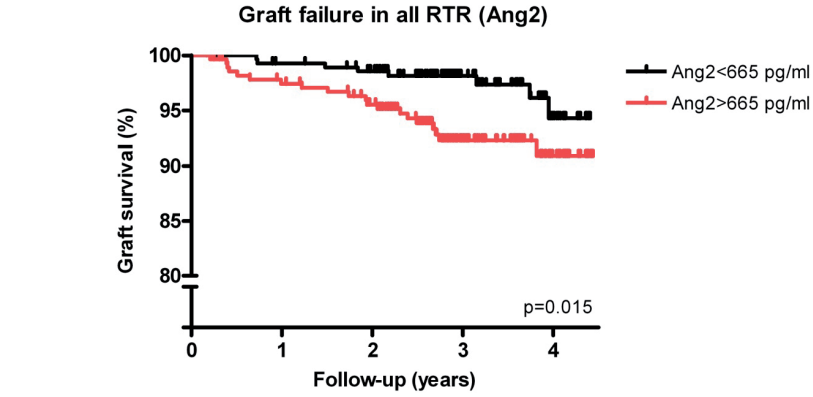
	Model 1		Model 2		Model 3		Model 4	
Dependent variable	β	p	β	p	β	p	β	p
Angiopoietin-1*								
SBP (mmHg)	0.04	0.27	0.05	0.20	0.04	0.23	0.04	0.28
DBP (mmHg)	0.07	0.04	0.07	0.04	0.07	0.05	0.07	0.09
MAP (mmHg)	0.04	0.26	0.05	0.19	0.05	0.21	0.05	0.25
Pulse pressure (mmHg)	-0.005	0.90	<0.001	0.99	-0.002	0.95	<0.001	0.99
Heart rate (bpm)	0.04	0.29	0.04	0.35	0.04	0.32	-0.008	0.84
Nt-pro-BNP* (ng/L)	0.01	0.78	0.03	0.27	0.03	0.32	-0.01	0.71
hsCRP* (mg/L)	0.04	0.34	0.04	0.28	0.04	0.30	-0.02	0.61
HbA1c* (%)	0.05	0.18	0.04	0.23	0.04	0.27	0.02	0.70
Angiopoietin-2*								
SBP (mmHg)	0.02	0.53	0.02	0.52	0.02	0.59	0.01	0.90
DBP (mmHg)	0.04	0.27	0.04	0.26	0.04	0.29	0.02	0.69
MAP (mmHg)	0.02	0.57	0.02	0.58	0.02	0.64	0.002	0.97
Pulse pressure (mmHg)	-0.003	0.94	-0.003	0.92	-0.007	0.85	-0.007	0.86
Heart rate (bpm)	0.12	0.001	0.12	0.001	0.13	0.001	0.13	0.001
Nt-pro-BNP* (ng/L)	0.14	<0.001	0.12	<0.001	0.12	<0.001	0.12	<0.001
hsCRP* (mg/L)	0.17	<0.001	0.16	<0.001	0.16	<0.001	0.17	<0.001
HbA1c* (%)	0.08	0.04	0.08	0.03	0.08	0.04	0.07	0.07

Coefficients are provided as standardized betas, referring to the number of standard deviations the dependent variable changes, per standard deviation increase of Ang1/Ang2.
Model 1: adjusted for age, sex
Model 2: additionally adjusted for eGFR and years since transplantation
Model 3: additionally adjusted for use of antihypertensives
Model 4: additionally adjusted for either Ang1 or Ang2
*Natural-logarithm transformation (LN) for analyses. Abbreviations: SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; Nt-pro-BNP, N-terminal pro-Brain Natriuretic Peptide; hsCRP, high-sensitive CRP; HbA1C, glycosylated hemoglobuline; eGFR, estimated glomerular filtration rate according to the Chronic Kidney Disease Epidemiology Collaboration

Association of Ang1 and Ang2 with death-censored graft failure

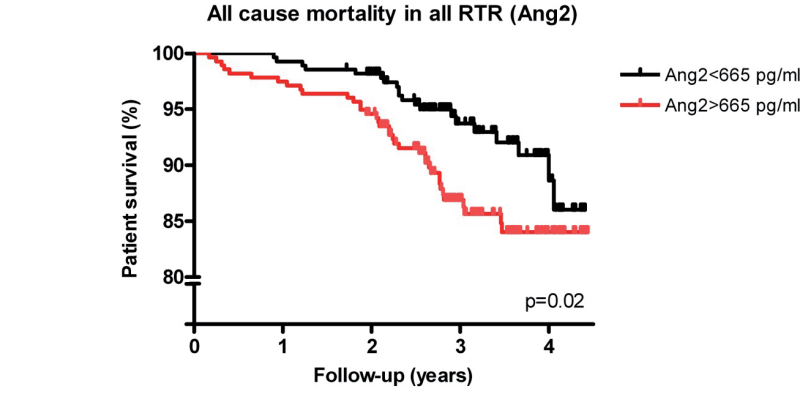
Median follow-up from baseline was 3.1 [IQR 2.6-3.8] years. During this follow-up 28 (5%) of 552 RTR developed graft failure. For Ang1, no association with graft failure was observed (Log-rank test p=0.17). Kaplan-Meier curves for death-censored graft failure stratified into Ang2 levels under vs. above the median (665 pg/ml) are shown in figure 1. Incidence of graft failure during follow-up in the low Ang2-group was 8 out of 276 (3%) vs. 20 out of 276 (7%) in the high Ang2-group. Ang2 levels above the median were significantly associated with death-censored graft failure (Log-rank test p=0.015). The association between plasma Ang1 and Ang2

levels with graft failure was assessed using Cox regression analysis (table 3). After adjustment for age and sex, the significant association between Ang2 levels and graft failure was lost.



No. at risk					
Ang2<665 pg/ml	276	273	262	133	38
Ang2>665 pg/ml	276	263	247	137	47

Figure 1. Kaplan-Meier curves and numbers at risk for death-censored graft survival of 552 renal transplant recipients (RTR). Survival rates stratified into high and low Ang2 level, under versus above the median of 665 pg/ml. Log-rank test p=0.015.



No. at risk					
Ang2<665 pg/ml	275	274	263	133	40
Ang2>665 pg/ml	277	271	260	149	51

Figure 2. Kaplan-Meier curves and numbers at risk for patient survival of 552 renal transplant recipients (RTR). Stratified into high and low Ang2 level, under versus above the median of 665pg/ml. Log-rank test p=0.02.

Table 3. Cox regression analyses for prediction of death-censored graft failure based on plasma Ang1 and Ang2 levels in 552 renal transplant recipients

	Angiopoietin-1*			Angiopoietin-2*		
	N_{total}= 552/ N_{events}= 28					
	HR	95% CI	p	HR	95% CI	p
Model 1	1.45	0.63-3.30	0.38	1.75	1.00-3.07	0.049
Model 2	1.32	0.57-3.05	0.51	1.71	0.95-3.08	0.07
Model 3	1.38	0.60-3.15	0.45	1.79	0.98-3.23	0.05
Model 4	1.70	0.56-5.19	0.35	2.02	0.97-4.23	0.06

*Angiopoietin-1 and angiopoietin-2 were natural-logarithmic (LN) transformed for analyses. Hazard ratios are associated with a 1-unit increase in each covariate. CI: Confidence Interval, HR: Hazard Ratio.
Model 1: crude model
Model 2: adjusted for age, sex and donor type
Model 3: model 2 plus adjustment for cold ischemia time, number of transplantations, number of mismatches
Model 4: model 3 plus adjustment for transplantation vintage, albuminuria and renal function (eGFR, CKD)

Association of Ang1 and Ang2 with all-cause mortality

During follow-up 59 (11%) of 552 RTR died. Kaplan-Meier curves for all-cause mortality stratified into Ang2 levels under vs. above the median (665 pg/ml) are shown in figure 2. For Ang1, no association with mortality was observed (Log-rank test p=0.46). Incidence of mortality during follow-up in the low Ang2-group was 22 out of 276 (8%) vs. 37 out of 276 (13%) in the high Ang2-group. Ang2 levels above the median were significantly associated with mortality (Log-rank test p=0.02). The association between plasma Ang1 and Ang2 levels with all-cause mortality was assessed in table 4 using Cox regression analysis. After adjustment for all possible confounders (model 4), Ang2 levels were significantly associated with mortality (p=0.045).

Secondary analysis with Ang2 according to donor type

In analyses stratified according to donor type, a difference in associations of plasma Ang2 with graft failure was observed (table 5). After adjustment for age, sex, cold ischemia time, number of transplantations, number of mismatches, years since transplantation, albuminuria and renal function (model 4), plasma Ang2 levels were significantly associated with graft failure in deceased donor-RTR (p=0.03). No association between plasma Ang1 and graft failure in the separate donor types was found. For all-cause mortality as well, a difference in associations of plasma Ang2 was observed in stratified analysis according to donor type (table 6). After adjustment for age, sex, diabetes, donor type, years since transplantation and renal function (model 4), plasma Ang2 levels were significantly associated with mortality in deceased donor-RTR (p=0.04). No association between plasma Ang1 and all-cause mortality in the separate donor types was found.

Table 4. Cox regression analyses for prediction of all-cause mortality based on plasma Ang1 and Ang2 levels in 552 renal transplant recipients

	Angiopoietin-1*			Angiopoietin-2*		
	N_{total}= 542/ N_{events}= 59					
	HR	95% CI	p	HR	95% CI	p
Model 1	1.03	0.61-1.74	0.90	1.73	1.16-2.58	0.007
Model 2	1.13	0.65-1.96	0.68	1.76	1.18-2.62	0.005
Model 3	1.09	0.64-1.86	0.76	1.52	1.05-2.21	0.03
Model 4	1.08	0.62-1.89	0.78	1.46	1.01-2.10	0.045

*Angiopoietin-1 and angiopoietin-2 were natural-logarithmic (LN) transformed for analyses. Hazard ratios are associated with a 1-unit increase in each covariate. CI: Confidence Interval, HR: Hazard Ratio.
Model 1: crude model
Model 2: adjusted for age and sex
Model 3: model 2 plus adjustment for donor type and diabetes
Model 4: model 3 plus adjustment for transplantation vintage and renal function (eGFR, CKD)

Table 5. Cox regression analyses for prediction of death-censored graft failure based on plasma Ang2 levels in 552 renal transplant recipients separated per donor type

	Angiopoietin-2*					
	Deceased donor			Living donor		
	N_{total}= 352/ N_{events} = 20			N_{total}= 200/ N_{events}= 8		
	HR	95% CI	p	HR	95% CI	p
Model 1	2.36	1.15-4.84	0.02	1.03	0.50-2.14	0.94
Model 2	2.38	1.10-5.13	0.03	1.13	0.54-2.39	0.75
Model 3	2.82	1.23-6.47	0.02	1.16	0.47-2.83	0.75
Model 4	3.64	1.17-11.28	0.03	0.98	0.34-2.81	0.96

*Angiopoietin-2 was natural-logarithmic (LN) transformed for analyses. Hazard ratios are associated with a 1-unit increase in each covariate. HR: Hazard Ratio; CI: Confidence Interval.
Model 1: crude model
Model 2: adjusted for age, sex
Model 3: model 2 plus adjustment for cold ischemia time, number of transplantations, number of mismatches
Model 4: model 3 plus adjustment for transplantation vintage, albuminuria and renal function (eGFR, CKD)

DISCUSSION

The major finding of this study is that Ang2 plasma levels are associated with death-censored graft failure and all-cause mortality after renal transplantation in a large cohort of kidney transplant recipients. The association between Ang2 levels and graft failure and mortality is independent of established risk factors. We additionally found higher plasma Ang2 levels in RTR who received a kidney from a deceased donor compared to those from RTR who received a kidney from a living

Table 6. Cox regression analyses for prediction of all-cause mortality based on plasma Ang2 levels in 552 renal transplant recipients separated per donor type

	Angiopoietin-2*					
	Deceased donor			Living donor		
	N_{total}= 353/ N_{events} = 50			N_{total}= 189/ N_{events} = 9		
	HR	95% CI	p	HR	95% CI	p
Model 1	1.75	1.15-2.66	0.009	1.08	0.45-2.60	0.86
Model 2	1.78	1.18-2.70	0.007	1.07	0.44-2.61	0.88
Model 3	1.61	1.08-2.41	0.02	0.96	0.39-2.39	0.93
Model 4	1.53	1.03-2.27	0.04	1.04	0.36-3.01	0.95

*Angiopoietin-2 was natural-logarithmic (LN) transformed for analyses. Hazard ratios are associated with a 1-unit increase in each covariate. CI: Confidence Interval, HR: Hazard Ratio.
Model 1: crude model
Model 2: adjusted for age and sex
Model 3: model 2 plus adjustment for diabetes
Model 4: model 3 plus adjustment for transplantation vintage and renal function (eGFR, CKD)

donor. In deceased donor-RTR, secondary analysis demonstrated an association of plasma Ang2 with both graft failure and mortality.

Our findings extend previous data in which Ang2 levels predicted mortality in CKD patients and critically ill patients[24,31]. To further underline the clinical importance of Ang2 in renal transplantation and different donor types, we here demonstrate its predictive value after renal transplantation in a large cohort of renal transplant recipients. The significance of increased Ang2 is also reflected by the identification of Ang2 as a promising biomarker in predicting severe acute pancreatitis[43] and associations between Ang2 and critical illness such as acute respiratory distress syndrome, myocardial infarction, sepsis and trauma[12]. To clarify the potential of clinically measuring Ang2 in the scope of CVD and mortality after renal transplantation, it should critically be compared to other clinical signs of CVD or predictors of mortality in future prospective studies to see if the Ang2 levels rise prior to the established signs.

Since deceased donors suffer from unfavorable inflammatory responses which cause endothelial activation, we compared the Ang1 and Ang2 levels between deceased donor-RTR and living donor-RTR. As anticipated, deceased donor-RTR have higher Ang2 levels compared to living donor-RTR. Whereas the Ang2 levels we found in the control and living donor-RTR group are quite similar to the levels measured in other healthy individuals[44].

Although early after transplantation a rapid Ang2 release has been observed by others, we did not find a difference in Ang2 levels between the whole RTR group and controls, possibly due to the longer time frame between transplantation and sample withdrawal in our study groups[45]. It is possible that the elevated Ang2 levels

we found in the deceased donor-RTR are a consequence of pre-existent damage caused during time of donation[39,45,46].

At this period of time after transplantation, the difference in vascular status between the RTR and controls may not be reflected by the rapidly responding Ang2 or hsCRP levels but possibly by the constant high expression of Ang1 which correlates to the magnitude of vascular resistance[47,48]. Our results show that the increased mortality risk among RTR is independent of Ang1 levels, supporting the hypothesis that Ang2 is the more dynamic player, reacting to donor type, of these two growth factors.

The difference in mortality we found for Ang2 levels under vs. above the median in Kaplan-Meier analyses of mortality and graft failure was further extended by Cox regression analyses, in which we adjusted for potential confounders. The crude association remained after these adjustments. These results are in line with those of a case control study, in which it was reported that serum Ang2 levels predict mortality in kidney transplant recipients[49].

Our findings point to an unfavorable role for Ang2 in the development and/or progression of a detrimental cardiovascular profile following renal transplantation. We found significant independent relationships of Ang2 with hsCRP and Nt-pro-BNP in our multivariate lineair regression analysis. This is similar to previous data in which Ang2 levels are positively associated with CRP in another cohort of renal transplant recipients[49]. Similar findings were done at the onset of COPD exacerbations[49,50]. Moreover, Ang2 levels are positively associated with hsCRP in patients with coronary heart disease[50,51].

A potential mechanistic explanation for our findings is that Ang2 is involved in the pathogenesis of vascular inflammation, endothelial activation and atherosclerosis. Plasma Ang2 expression is increased in atherosclerotic plaques and correlates with plaque microvascular density and matrix metalloproteinase 2 (MMP-2) activity[52]. Ang2 has also been identified as a link between kidney fibrosis and arterial stiffness since blockade attenuated expression of monocyte chemokines, profibrotic cytokines and collagen in aorta of mice after 5/6 subtotal nephrectomy[53]. Although the expression of Ang2 has been known to be tightly controlled and is strongly increased following stimulation by cytokines, growth factors and environmental factors, the mode of Ang2 secretion by WPB has never been established[2,4,54]. However, there is evidence that this process is inhibited by the PI3K/Akt/endothelial nitric oxide synthase (eNOS) signaling pathway, which plays an important role in vascular growth and stabilization[55]. The physiological basis for the association between elevated circulating Ang2 and graft failure remains to be unraveled. In acute settings like pancreatitis, angiopoietins play a role in the first hour of the inflammation process, whereas their long-term effects are associated with vascular remodeling[44]. Even though the exact mechanism has not yet been completely elucidated, the angiopoietin/Tie2 ligand-receptor

system seems to have a distinct regulatory function in acute inflammation and structural remodelling. The resting endothelium expresses Ang2 weakly and stores Ang2 in WPB from where it can be made available quickly following stimulation, suggesting a role of Ang2 in controlling rapid vascular adaptive processes[4]. In adult mice and human, Ang2 is only expressed at sites of vascular remodelling[2]. Possibly RTR bear a pre-inflammatory state which aggravates in case there is a risk for organ failure, reflected by increased Ang2-supported endothelial activation[12,56]. Many studies have focused on anti-Ang2 therapy in the treatment of chronic rejection in rat cardiac allografts, malignant tumors including monoclonal antibodies and siRNAs[18-20,57,58]. None of them is evaluated in the setting of renal transplantation, although targeting Ang2 to attenuate inflammation may provide a novel therapy for graft failure and patient mortality in RTR, especially in the setting of deceased donation.

Several limitations of our study deserve acknowledgement. This study is an observational epidemiologic study, which makes it difficult to draw conclusions on causality. In general, statistical significance in observational studies suggests, but does not confirm, biologic significance. Whether the significant relation between Ang2 and mortality in RTR is a causal or an associative relation remains to be determined. However, the association between Ang2 and mortality and graft failure remained significant in multivariate Cox regression analyses, including renal function, suggesting that this association is independent of confounding factors. In addition, our study population was enrolled from a single center and was predominantly Caucasian which limits the generalizability of this study. Second, specific indices of vascular inflammation and potential subclinical atherosclerosis, as intermediate endpoints, were not assessed in this cohort. The Ang1 concentration we measured might be explained by variation in sample processing. Unlike Ang2, Ang1 is not exclusively expressed by the vascular wall. A high amount of Ang1 is also found in platelets[59]. False positive Ang1 levels can therefore result from the *ex vivo* activation of platelets in serum tubes[7]. Another possibility for the increased Ang1 levels could be the use of immunosuppressive treatment, most probably steroid treatment[60].

It is an observational study of well defined RTR with a large sample size and complete follow-up. Extensive data collection, including data from 24h urine samples allowed for adjustment for many potential confounders in our analysis. We speculate that targeting the Ang/Tie2-system, more specifically Ang2, may be a strategy to reduce the observed detrimental associations of Ang2 with patient and graft survival, especially after deceased donation. However, more studies investigating the cause of Ang2 release and its effects are needed to clarify the role of the angiopoietin/Tie2-system in renal transplantation.

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