Angiopoietins in renal replacement therapy

Westendorp, Welmoet Hillegonda

CHAPTER 2

Angiopoietin-2 associates with markers of inflammation and cardiac damage and predicts cardiovascular events and mortality in dialysis patients

WH Westendorp
S Assa
H van Goor
R Westerhuis
SJL Bakker
HGD Leuvenink
CFM Franssen

Submitted

ABSTRACT

Background

Angiopoietin-1 (Ang1) and Angiopoietin-2 (Ang2) are involved in inflammation and vascular stabilization. Elevated Ang2 expression is caused by endothelial activation and may play a role in arteriosclerosis, cardiovascular disease (CVD) and mortality in chronic kidney disease (CKD). We studied the association of Ang1 and Ang2 with markers of inflammation, fluid overload, cardiac damage, clinical parameters and outcome in a prospective cohort of prevalent hemodialysis (HD) patients.

Study design and analytical approach

Plasma Ang1 and Ang2 were measured in 100 patients in a single HD session on four different time points: predialysis, at 60 and 180 min intradialysis and postdialysis (240 min). Cross-sectional analyses were performed through uni- and multivariable linear regression models and prospective analyses through uni- and multivariable Cox-regression models.

Candidate predictors

Ang1 and Ang2.

Outcomes

Cardiovascular events and all-cause mortality.

Results

Ang1 decreased from pre-HD to post-HD ($p=0.001$), whereas Ang2 levels rose markedly during the first hour of HD (from 2.6 (IQR 1.6-4.5) to 3.7 ng/ml (IQR 2.4-5.4), $p=0.005$) and subsequently decreased to values that were comparable with pre-HD values (2.5 ng/ml (IQR 1.9-3.6). For Ang1, no association with hsCRP, IL-6, TNFα, pentraxin-3, myeloperoxidase, pro-endothelin, ultrafiltration rate, Nt-pro-BNP and cardiac troponin T was observed. Similar analysis demonstrated associations with Ang2, independent of potential confounders (all $p<0.05$). At all time points, Ang2 levels associated with cardiovascular events (all <0.05). A greater intra-HD rise in Ang2 was associated with all-cause mortality (HR 1.2, 95%CI 1.2-1.2, $p<0.001$) independent of possible confounders. For Ang1, no such associations were found.

Conclusion

Ang2 is associated with inflammation and cardiac damage. Higher Ang2 levels are associated with a higher incidence of all-cause mortality and cardiovascular events in dialysis patients. HD treatment per se induces acute changes in angiopoietin levels suggestive of systemic endothelial activation. Greater intra-HD increases in Ang2 are associated with higher mortality. It remains to be studied whether intervention by Ang2 inhibitors attenuates the HD-induced angiopoietin-disequilibrium and improves outcome.

INTRODUCTION

Angiopoietin-1 (Ang1) and angiopoietin-2 (Ang2), ligands of the Tie2 receptor and endothelial growth factors, are involved in stabilizing the vascular endothelium. Ang1 mediated Tie2 signaling promotes structural integrity of blood vessels and maintains quiescent endothelium[1,2]. Ang2 is classically considered as a Tie2 antagonist, counteracting the stabilizing effects of Ang1 in a dose-dependent manner and priming the endothelium to respond to exogenous pro-inflammatory stimuli[3,4]. An abnormal Ang1/Ang2 ratio, with downregulation of Ang1 and upregulation of Ang2 has been observed in CVD[5-7]. In clinical studies, elevated Ang2 levels were associated with peripheral artery disease[8] and heart failure[9,10]. In the general population, Ang2 levels predict cardiovascular morbidity[11]. Moreover, Ang2 expression is increased in plaque neovascularization, supporting the notion that endothelial activation is involved in the pathogenesis and progression of vascular inflammation and atherosclerosis[12,13].

Accelerated atherosclerosis in patients with CKD is still incompletely understood, but accumulating evidence suggests that Ang1 and Ang2 are important factors. Ang2 levels are elevated in patients with chronic kidney disease (CKD) and have a positive correlation with albuminuria and predict long-term mortality in this population[14-16]. In patients on hemodialysis (HD), Ang2 levels are markedly elevated and correlate with the severity of atherosclerotic lesions[12,15,17].

Ang1 is mainly synthesized in periendothelial cells, including vascular smooth muscle cells, pericytes and astrocytes. In adult vessels, Ang1 is constitutively expressed[18], while Ang2 expression is only observed at sites of active vascular remodeling and neoangiogenesis. Following endothelial activation, Ang2 is instantly released by exocytosis of endothelial Weibel Palade bodies (WPB)[19-21].

Although HD is life-saving in patients requiring renal replacement therapy, the HD procedure itself may contribute to the chronic inflammatory state of these patients. HD is capable of inducing various inflammatory pathways, mainly as a result of contact between the blood and the extra-corporal system, regardless of the dialyser type[22-24]. During HD, leukocyte activation is evidenced by increases in rapid-reacting cytokines like pentraxin-3[25], increased leukocyte transcript levels of several pro-inflammatory cytokines such as TNF-α and IL-8[26] and a rise in myeloperoxidase (MPO) levels during HD[27]. The HD-related systemic inflammation and oxidative stress may well contribute to the endothelial dysfunction of HD patients[28,29]. Ang2 might be a mediator and/or marker for inflammation, endothelial function, and accelerated atherosclerosis in dialysis patients. Furthermore, Ang2 could be a potential new therapeutic target to improve outcome by reducing the endothelial response to the chronic inflammatory state in CKD patients as well as the acute HD-induced inflammation. This is highly relevant since it is possible to influence Ang2 levels via Ang2 inhibitors[30,31].

In this study we tested the hypothesis that angiopoietin levels in dialysis patients are associated with higher levels of markers of inflammation, endothelial dysfunction, volume overload and cardiac damage. Additionally, we studied whether the HD procedure itself affects angiopoietin levels and if pre-, intra- and postdialysis Ang1 and Ang2 are associated with outcome.

METHODS

Patients and Study Design

For this study, we analyzed Ang1 and Ang2 levels in stored plasma samples of patients that participated in a prospective observational single-center cohort study[25]. Adult HD patients (aged ≥18 years) from the Dialysis Center Groningen and the University Medical Center Groningen were eligible if they had been treated with HD for more than 3 months and were on a thrice-weekly dialysis schedule. A total of 109 out of 235 in-center patients signed written informed consent. For the current post-hoc analysis, plasma Ang1 and Ang2 levels were measured in patients in which there were complete data and sufficient plasma sample volumes to measure Ang1 and Ang2 (n = 100, 91.7%). The study was performed according to the Declaration of Helsinki and the study protocol was approved by the Institutional Review Board of the University Medical Center Groningen (METc 2008.343). The study was performed between March 2009 and March 2010. Plasma Ang1 and Ang2 were measured in December 2013. Patients were studied at the dialysis session after the longest interdialytic interval (3 days).

HD session length was 4 hours. Patient characteristics were assessed at study entry from medical records. Diabetes was defined as fasting blood glucose level >6 mmol/L or use of antidiabetic drugs. Hypertension was defined as predialysis systolic blood pressure >140 mmHg and/or diastolic blood pressure >90 mmHg or use of antihypertensive drugs. Cardiovascular history was defined as any history of ischemic heart disease, congestive heart failure, coronary artery bypass graft, percutaneous coronary intervention, stroke, or peripheral vascular disease. Blood pressure and heart rate were measured before, during and after HD. Ultrafiltration rate was expressed in ml/kg/h by dividing the ultrafiltration volume by dialysis session length and target weight[32]. Equilibrated Kt/V was calculated from pre- and postdialysis plasma urea concentration according to the second-generation logarithmic Daurgirdas equation[33].

Dialysis settings

All patients were on bicarbonate HD with a low-flux polysulfone hollow-fiber dialyzer (F8; Fresenius Medical Care). Blood flow and dialysate flow rates were 250-350 and 500 ml/min, respectively. Dialysate temperature was 36.0°C in all patients. Dialysate composition was as follows: sodium 139 mmol/L; calcium 1.5 mmol/L;

magnesium 0.5 mmol/L; chloride 108 mmol/L; bicarbonate 34 mmol/L; acetate 3.0 mmol/L; and glucose 1.0 g/L. Potassium concentration was 1.0 or 2.0 mmol/L, depending on prevailing plasma potassium concentrations. We used constant ultrafiltration rate and dialysate conductivity. The water for hemodialysis complied with the requirements of the European Pharmacopoeia (<100 colony-forming units/ml; <0.25 endotoxin units/ml). Patients received a light meal at 60 minutes intradialysis. Patients received dialysis in a supine position, which excluded the effect of posture changes on blood volume.

Laboratory procedures

Blood samples were collected from the arterial line of dialysis circuit at the start of HD, 60 and 180 minutes intra-HD, and at the end of dialysis. Hematocrit, leukocytes, neutrophils, albumin, calcium, and phosphate were determined immediately. For the determination of cytokines, blood was centrifuged within 30 min of collection at 3500 rpm for 15 min. Supernatants were stored at -80°C until measurement. Prior to assay, samples were thawed and recentrifuged. Samples were analyzed at a single time point to eliminate inter-assay variability.

Plasma parameters

High-sensitive CRP (hsCRP) was measured with N latex CRP monoassay (Siemens Diagnostic, Newark, DE, USA). Pentraxin-3 and interleukin-6 (IL-6) were measured by quantitative sandwich enzyme immunoassay technique (R&D Systems, Minneapolis, USA). Pentraxin-3 was measured because it responds rapidly to inflammatory stimuli and is considered as an appropriate marker for investigating inflammatory reactions that may occur during single dialysis sessions[34]. Tumor necrosis factor α (TNFα) was measured by Quantikine HS Human Immunoassay (R&D Systems, Minneapolis, USA). Myeloperoxidase, which reflects activation of leukocytes, was measured by ELISA (Hytest, Turku, Finland). Pro-endothelin was measured by novel sandwich fluoroimmunoassay (BRAHMS, Hennigsdorf/Berlin, Germany) using the automated system B.R.A.H.M.S KRYPTOR. Measurement of endothelin was based on competition with surface-bound recombinant endothelin (RayBiotech, Norcross, GA, USA) for binding to a specific antibody (RayBiotech). The amount of captured antibody was measured by HRP-labeled secondary antibody and subsequent substrate conversion. Plasma Ang1 and Ang2 levels were measured via enzyme-linked immunosorbent assay (ELISA) Duosets (R&D Systems, Minneapolis, USA). Concentrations of all biomarkers measured during and after dialysis were corrected for the effect of hemoconcentration according to Schneditz et al[35].

Clinical endpoints

The primary endpoint of this study was all-cause mortality during a follow up period of three years. The secondary outcome was cardiovascular events defined as the

occurrence of ischemic heart disease, congestive heart failure, coronary artery bypass graft, percutaneous coronary intervention, stroke, or peripheral vascular disease. Patients were censored at the time of renal transplantation. Data endpoints regarding survival and cardiovascular events were obtained from hospital charts. None of the patients was lost to follow-up.

Statistical analysis

Data are reported as mean ± SD (standard deviation) for continuous variables with normal distributions, median [interquartile range] for skewed variables, and number (%) for categorical data. The Kruskal-Wallis test was used to assess whether angiopoietin levels were significantly different among the four time points. Subsequently, significant differences of Ang2 between two time points were tested using the Mann-Whitney test. Differences between patients with an increase versus a decrease in angiopoietins during HD were analyzed with Chi-square test in case of dichotomous variables and Mann-Whitney in case of continues variables. Skewed data were normalized for analyses by natural-logarithm transformation (LN) transformation. The associations of Ang1 and Ang2 levels with various clinical parameters were analyzed with crude linear regression analysis (model 1), with adjustment for age, sex, dialysis vintage (model 2), and with additional adjustment for diabetes, cardiovascular history and ultrafiltration volume (model 3). Regression coefficients are given as standardized betas. The same models were used to perform prospective Cox regression analyses for the association of angiopoietins with all-cause mortality and cardiovascular events. In the analyses of intradialytic Ang2 changes, additional adjustment was performed for predialysis Ang2 level (model 4). In the analyses of intradialytic Ang2 change, hazard ratios are given per standard deviation multiplied with 100. Two-sided P value <0.05 was considered significant. Statistical analyses were performed with SPSS version 20 (SPSS Inc. Chicago, USA).

RESULTS

Patient characteristics and angiopoietin concentrations

Baseline characteristics of the 100 patients eligible for angiopoietin analyses are shown in table 1. The median (IQR) age was 66 (50-75) years. Twenty-four patients had diabetes and 81 used antihypertensive medication. Twenty-three patients had a history of cardiovascular events. The course of Ang1 concentrations during HD is shown in figure 1a. Ang1 gradually decreased from 2.8 ng/ml (IQR 1.8-5.0) predialysis to 2.7 ng/ml (IQR 1.8-3.9) at 60 min intra-HD (p=0.36), 2.6 ng/ml (IQR 1.6-4.0) at 180 min intra-HD (p=0.47) and 2.2 ng/ml (IQR 1.5-2.9) post-HD. Post-HD Ang1 levels were significantly lower compared with predialysis levels (p=0.001). The course of Ang2 concentrations is shown in figure 1b. Ang2 medians differed significantly among the four time points (p=0.001). Ang2 increased significantly

Table 1. Baseline patient characteristics and predialysis biomarker concentrations

	Patients on HD n = 100
Age, years	66 [50-75]
Males, %	67
Dialysis vintage, years	1.7 [0.7-4.0]
Diabetes, %	24
Hypertension, %	81
Predialysis SBP, mmHg	141 ± 25
Predialysis DPB, mmHg	81 ± 17
Heart rate, BPM	75 ± 14
Body mass index, kg/m^2	25 [23-28]
Body surface area, m^2)	1.9 ± 0.2
Previous cardiovascular events, %	23
Ultrafiltration volume, ml	2578 ± 773
Ultrafiltration rate, ml/kg/h	8.6 ± 2.6
Hematocrit, %	35 ± 3.7
Albumin, g/L	39 [37-40]
Calcium, mmol/L	2.3 ± 0.2
Phosphate, mmol/L	1.6 [1.3-1.9]
Kt/V	4.2 [3.8-4.9]
Angiopoietin-1, ng/ml	2.8 [1.8-5.0]
Angiopoietin-2, ng/ml	2.6 [1.6-4.5]
hsCRP, mg/L	6.9 [2.9-13.8]
Nt-pro-BNP, ng/L	4 [1.7-8.5]
Cardiac Troponin T, ng/L	46.3 [27-81.1]
Cardiac Troponin I, ng/L	0.02 [0.01-0.03]
Pentraxin-3, ng/ml	2.6 [1.6-4.2]
Myeloperoxidase, ng/ml	84.7 [73.1-98.3]
IL-6, pg/ml	6.1 [4-8.6]
TNFα, pg/ml	3.4 [2.8-4.2]
Pro-endothelin, pmol/L	275 [224-330]
Endothelin, ng/ml	40 [22.5-63]
HbA1C, %	5.7 ± 1

Data are presented as mean ± SD in case of normal distribution and as median [interquartile range] in case of skewed distribution. IQR, interquartile range; n, number; HD, hemodialysis; SBP, systolic blood pressure; DBP, diastolic blood pressure; BPM, beats per minute; hsCRP, high-sensitive CRP; Nt-pro-BNP, N-terminal pro-Brain Natriuretic Peptide; IL-6, interleukin-6; TNF-α, tumor necrosis factor α; HbA1C, glycosylated hemoglobin.

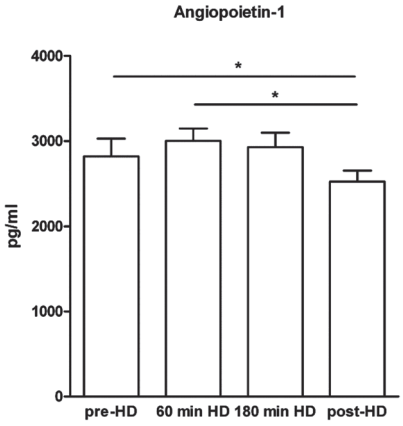

Figure 1a. Pre-, intra- and postdialysis Ang1 levels. Error bars indicate mean±SD of 100 patients. Postdialysis Ang1 levels were significantly lower compared with predialysis (p=0.001) and 60 min intradialysis (p=0.01).

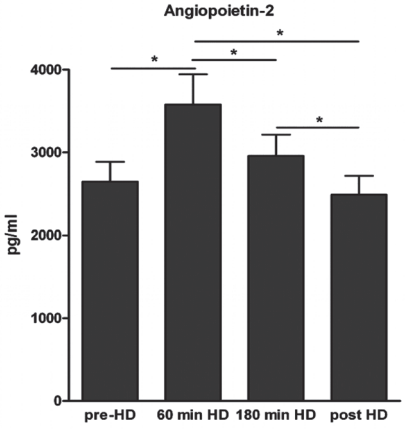

Figure 1b. Pre-, intra- and postdialysis Ang2 levels. Error bars indicate mean±SD of 100 patients. Ang2 levels increased significantly from predialysis to 60 min intradialysis (p=0.005). Pre- and post-HD Ang2 levels did not differ significantly (p=0.50).

from 2.6 ng/ml (IQR 1.6-4.5) predialysis to 3.7 ng/ml (IQR 2.4-5.4, p=0.005) at 60 min intra-HD. Subsequently, Ang2 decreased to 3.0 ng/ml (IQR 2.1-4.1) at 180 min intra-HD (p=0.02, compared to 60 min intra-HD) and 2.5 ng/ml (IQR 1.9-3.6) post-HD (p=0.04, compared to 180 min intra-HD). Pre- and post-HD Ang2 levels did not differ significantly (p=0.50).

Associations of predialysis Ang1 and Ang2 with clinical parameters and laboratory markers

Associations with predialysis Ang1 and Ang2 levels are shown in table 2. In the crude model, predialysis Ang1 levels were associated with higher systolic blood pressure (β 0.22 p=0.03), diastolic blood pressure (β 0.22 p=0.03) and cardiac troponin I (β 0.25 p=0.02). The associations with diastolic blood pressure and cardiac troponin I remained after further adjustment. Pre-HD Ang2 levels were associated with markers of inflammation, including hsCRP (β 0.21, p=0.04), IL-6 (β 0.40, p<0.001), TNFα (β 0.38, p<0.001), pentraxin-3 (β 0.33, p=0.001), myeloperoxidase (β 0.27, p=0.008), pro-endothelin (β 0.43, p<0.001), and cardiac damage (Nt-pro-BNP, β 0.52, p<0.001; cardiac troponin T, β 0.32, p=0.002) in the crude model. These associations remained materially unchanged after further adjustment.

Association between Ang1 and long-term outcome on dialysis

Median follow-up from baseline was 32.8 (IQR 15.9-36.5) months. During this follow-up, thirty-six (36%) patients died and thirty-five (35%) patients had at least one cardiovascular event. For Ang1, there was neither an association with all-cause mortality nor with cardiovascular events (supplementary table 1).

Association between Ang2 and long-term outcome on dialysis

The multivariate association of plasma Ang2 and outcome is shown in table 3. Predialysis Ang2 did not associate with mortality (HR 1.3, 95%CI 0.8-2.1, p=0.37, crude model). However, higher Ang2 levels were associated with higher mortality at 180 min intra HD (HR 4.5, 95%CI 1.9-10.5, p=0.001) and post-HD (HR 4.6, 95%CI 2.1-10.3, p<0.001), independent of age, sex, dialysis vintage, diabetes, cardiovascular history and ultrafiltration volume (model 3). Higher Ang2 levels were associated with a significantly higher incidence of cardiovascular events at all time points: pre-HD: HR 1.8, 95%CI 1.1-3.2, p=0.04, 60 min intra-HD: HR 1.9, 95% 1.1-3.4, p=0.03; 180 min intra-HD: HR 3.5, 95% 1.7-7.2, p<0.001 and post-HD: HR 3.3, 95%CI 1.7-6.4, p=0.001 in the fully adjusted model (model 4).

Association between the intradialytic change in Ang1 and Ang2 and long-term outcome on dialysis

As Ang1 levels decreased significantly from pre- to post-HD, we analyzed whether Ang1 change during the HD session was predictive of outcome. The change in Ang1 was not predictive of all-cause mortality or cardiovascular events (supplementary table 2). In patients with an Ang1 increase during HD (n=64), Ang1 rose from 1.9 ng/ml (IQR 1.4-2.5) pre-HD to 2.5 ng/ml (IQR 1.9-3.7) post-HD. In patients with a stable or decrease in Ang1 levels (n=36), Ang1 decreased from 3.6 ng/ml (IQR 2.3-5.4) pre-HD to 2.1 ng/ml (IQR 1.4-2.9) post-HD. All-cause mortality (Log-rank test p= 0.13) and cardiovascular events (Log-rank test p=0.29) did not differ between the 2 groups.

Table 2. Regression coefficients for the association of predialysis plasma Ang1 and Ang2 with various clinical and laboratory parameters in 100 hemodialysis patients

	Model 1		Model 2		Model 3	
Dependent variable	β	p	β	p	β	p
Angiopoietin-1*						
SBP (mmHg)	0.22	**0.03**	0.21	0.05	0.20	0.06
DBP (mmHg)	0.22	**0.03**	0.26	**0.02**	0.28	**0.02**
Heart rate (beats/min)	0.05	0.60	0.06	0.60	0.06	0.60
Ultrafiltration rate (ml/kg/h)	-0.05	0.62	-0.06	0.53	-0.06	0.55
Ultrafiltration volume (L)	-0.11	0.28	-0.09	0.30	-0.09	0.30
HbA1C (%)*	-0.008	0.94	-0.02	0.84	-0.02	0.88
hsCRP (mg/L)*	-0.03	0.78	-0.02	0.88	-0.03	0.78
IL-6 (pg/ml)*	-0.11	0.30	-0.11	0.29	-0.10	0.36
TNFα (pg/ml)*	0.01	0.95	-0.009	0.94	-0.02	0.88
Nt-pro-BNP (ng/ml)*	0.12	0.25	0.11	0.31	0.14	0.20
Cardiac troponin T (ng/L)*	0.12	0.23	0.17	0.15	0.24	0.05
Cardiac troponin I (ng/L)*	0.25	**0.02**	0.26	**0.03**	0.29	**0.02**
Pentraxin-3 (ng/ml)*	-0.01	0.91	-0.02	0.83	<0.001	0.99
Myeloperoxidase (ng/ml)*	0.19	0.07	0.19	0.07	0.19	0.08
Pro-endothelin (pmol/L)*	0.02	0.88	-0.001	0.99	0.03	0.81
Endothelin (ng/ml)	0.07	0.49	0.06	0.56	0.05	0.66
Angiopoietin-2*						
SBP (mmHg)	-0.03	0.74	-0.05	0.63	-0.06	0.60
DBP (mmHg)	-0.07	0.50	-0.05	0.67	-0.05	0.65
Heart rate (beats/min)	0.13	0.23	0.15	0.15	0.14	0.18
Ultrafiltration rate (ml/kg/h)	0.16	0.11	0.20	**0.03**	0.20	**0.04**
Ultrafiltration volume (L)	0.08	0.44	0.15	0.09	0.14	0.12
HbA1C (%)*	0.07	0.52	0.08	0.51	-0.03	0.84
hsCRP (mg/L)*	0.21	**0.04**	0.23	**0.04**	0.22	**0.04**
IL-6 (pg/ml)*	0.40	**<0.001**	0.39	**<0.001**	0.37	**0.001**
TNFα (pg/ml)*	0.38	**<0.001**	0.38	**0.001**	0.38	**0.001**
Nt-pro-BNP (ng/ml)*	0.52	**<0.001**	0.54	**<0.001**	0.54	**<0.001**
Cardiac troponin T (ng/L)*	0.32	**0.002**	0.39	**0.001**	0.38	**0.001**
Cardiac troponin I (ng/L)*	0.19	0.08	0.21	0.08	0.18	0.14
Pentraxin-3 (ng/ml)*	0.33	**0.001**	0.31	**0.003**	0.31	**0.003**
Myeloperoxidase (ng/ml)*	0.27	**0.008**	0.27	**0.01**	0.26	**0.01**
Pro-endothelin (pmol/L)*	0.43	**<0.001**	0.47	**<0.001**	0.45	**0.001**
Endothelin (ng/ml)	0.07	0.49	0.04	0.73	0.08	0.48

*Natural-logarithmic (LN) transformed for analyses. Coefficients are provided as standardized betas.
SBP, systolic blood pressure; DBP, diastolic blood pressure; Nt-pro-BNP, N-terminal pro-Brain Natriuretic Peptide; hsCRP, high-sensitive CRP; HbA1C, glycosylated hemoglobin.
Model 1: crude model
Model 2: adjusted for age, sex and dialysis vintage
Model 3: as model 2 + adjusted for diabetes, cardiovascular history and ultrafiltration volume (except in case of ultrafiltration volume and ultrafiltration rate as dependent variable)

Table 3. Cox regression analyses for prediction of outcome based on Ang2 levels

	All-cause mortality											
	Ang2 pre-HD*			Ang2 60 min intra-HD*			Ang2 180 min intra-HD*			Ang2 post-HD*		
	HR	95% CI	p	HR	95% CI	p	HR	95% CI	p	HR	95% CI	p
Model 1	1.3	0.8-2.1	0.37	1.5	0.9-2.7	0.12	2.2	1.1-4.5	0.02	2.4	1.3-4.5	0.005
Model 2	1.4	0.8-2.4	0.22	1.5	0.9-2.5	0.16	2.6	1.3-5.3	0.008	3.0	1.5-5.7	0.001
Model 3	1.6	0.9-2.8	0.14	1.6	0.9-2.8	0.10	4.5	1.9-10.5	0.001	4.6	2.1-10.3	<0.001
	Cardiovascular events											
Model 1	1.9	1.2-3.3	0.01	2.3	1.3-4.2	0.004	3.4	1.8-6.3	<0.001	3.5	2.0-6.2	<0.001
Model 2	2.1	1.3-3.7	0.005	2.3	1.3-4.0	0.005	3.5	2.0-6.3	<0.001	3.7	2.1-6.3	<0.001
Model 3	1.8	1.1-3.2	0.04	1.9	1.1-3.4	0.03	3.5	1.7-7.2	<0.001	3.3	1.7-6.4	0.001

*Ang2 was natural-logarithmic (LN) transformed for analyses. Hazard ratios are associated with a 1-unit increase in each covariate. Ang2: angiopoietin-2, CI: Confidence Interval, HR: Hazard Ratio.
Model 1: crude model
Model 2: adjusted for age, sex and dialysis vintage
Model 3: as model 2 + adjusted for diabetes, cardiovascular history and ultrafiltration volume

Table 4. Cox regression analyses for prediction of outcome based on the intra-HD change in Ang2 concentration

	Δ% Ang2 from pre-HD to post-HD*					
	All-cause mortality			Cardiovascular events		
	HR	95% CI	p	HR	95% CI	p
Model 1	1.2	1.2-1.2	0.008	1.2	1.2-1.2	0.95
Model 2	1.2	1.2-1.2	0.01	1.2	1.2-1.2	0.95
Model 3	1.2	1.2-1.2	0.006	1.2	1.2-1.2	0.96
Model 4	1.2	1.2-1.2	<0.001	1.2	1.2-1.2	0.49

*Concentrations were corrected for the effect of hemoconcentration. Hazard ratios per standard deviation multiplied with 100. CI: Confidence Interval, HR: Hazard Ratio.
Model 1: crude model
Model 2: adjusted for age, sex and dialysis vintage
Model 3: as model 2 + adjusted for diabetes, cardiovascular history and ultrafiltration volume
Model 4: as model 3 + adjusted for predialysis Ang2

Since the associations between higher Ang2 levels and higher all-cause mortality and cardiovascular events were stronger in the second half of the HD session, we also analyzed whether the change in Ang2 during HD was predictive of outcome. As shown in table 4, a greater intradialytic rise in Ang2 was associated with higher all-cause mortality in all models (HR 1.2, 95%CI 1.2-1.2, p<0.001). For cardiovascular events, no such association was found (table 4). In patients with an Ang2 increase (n=54), Ang2 rose from 1.9 ng/ml (IQR 1.2-3.2) pre-HD to 2.5 ng/ml (IQR 1.6-3.8) post-HD. In the remainder of patients, Ang2 levels were stable or decreased. In these patients, Ang2 levels decreased from 3.6 ng/ml (IQR 2.6-5.5) pre-HD to 2.5 ng/ml (IQR 2.0-3.5) post-HD. Patients whose Ang2 levels rose during HD had higher ultrafiltration volume (p=0.04) but lower predialysis Nt-pro-BNP levels (p=0.01) and lower predialysis Ang2 levels (p<0.001) compared with patients whose Ang2 levels decreased during HD (supplementary table 3).

DISCUSSION

The main findings of this study are that Ang2 levels are significantly associated with markers of inflammation, fluid overload and cardiac damage and that higher Ang2 levels are associated with a higher incidence of all-cause mortality and cardiovascular events. HD treatment induced acute changes in angiopoietin levels suggestive of endothelial activation and greater intra-HD increases in Ang2 were associated with higher mortality.

Only a few studies have investigated angiopoietins in HD patients[15,36]. We found slightly lower pre-HD Ang1 and Ang2 levels compared with another adult dialysis population, possibly as a result of the use of different assays[37]. Compared to serum

Ang1 and Ang2 in children on dialysis, we measured somewhat lower Ang1 and Ang2 plasma levels, perhaps caused by *ex vivo* activation of platelets in serum tubes and intra-essay differences between plasma and serum[38,39]. We did not include a control group with normal renal function but David et al previously found that patients on hemodialysis have lower Ang1 and higher Ang2 levels compared with healthy individuals[40]. In only 2 previous studies, Ang2 levels were measured before and after HD showing that predialysis and postdialysis Ang2 levels did not differ significantly[41,40]. Our study confirms the results of these studies but for the first time it shows that Ang2 levels peak after one hour of HD and subsequently gradually decrease to values that are comparable with predialysis levels.

Interestingly, Ang2 levels associated with various prognostically unfavorable factors like inflammation (hsCRP, pentraxin-3, IL-6, TNFα, myeloperoxidase, pro-endothelin), fluid overload (ultrafiltration rate, Nt-pro-BNP), and cardiac damage (cardiac troponin I). This may suggest that Ang2 has a central role in the pathogenesis of accelerated arteriosclerosis in HD patients. Non-hemorrhagic and hemorrhagic atherosclerotic plaques release Ang2[42]. The finding that Ang2 levels are elevated in patients with diabetes, CKD and atherosclerosis and correlate with vascular inflammation and disease progression supports the notion that elevated Ang2 levels reflect endothelial dysfunction[3,7,43]. The pro-inflammatory role of Ang2 in cardiovascular disease and atherosclerosis, has been demonstrated in previous studies[12,15,36,44]. The significant association between circulating Ang2 and hsCRP we demonstrated is consistent with previous reports[16,45]. Additionally, we found a significant association between Ang2 and the pro-inflammatory markers Il-6, TNF-alpha, myeloperoxidase, and pentraxin-3. Higher levels of these inflammatory markers are associated with a strongly elevated risk of cardiovascular events and mortality in patients on hemodialysis[46-48].

Since patients with CKD including those on maintenance HD are characterized by endothelial dysfunction[49,50] and since endothelial dysfunction is a known cause of Ang2 release[51,52], it is tempting to speculate that elevated predialysis Ang2 levels reflect endothelial dysfunction. This may also explain the strong association between Ang2 and pro-endothelin levels that we found. We observed a remarkable increase in Ang2 levels during the first hour of HD. The HD procedure may acutely worsen endothelial function as has been shown in children and adults[49,50]. Thus, the early rise of Ang2 during HD may reflect the deleterious effect of HD on the endothelium. HD acutely induces an inflammatory response as evidenced by significant intradialytic increases in rapidly reacting proinflammatory cytokines such as pentraxin-3[29,53]. The HD procedure is also associated with leukocyte activation and degranulation, which results in an acute intra-dialytic aggravation of oxidative stress[50]. Together, the inflammatory response and oxidative stress probably result in worsening of endothelial function during HD[50,54]. The early intradialytic rise in Ang2 levels may well reflect WPB exocytosis of Ang2 as a result of HD-induced inflammation and oxidative stress[21].

Patients with cardiovascular disease are more likely to have higher circulating Ang2 levels[6,9] compared with healthy controls. In the general population, elevated Ang2 levels are associated with an increased cardiovascular events and mortality[11]. Circulating Ang2 has also been shown to correlate with time on dialysis, systolic blood pressure and carotid artery intima media thickness in children with CKD on dialysis[36]. In line with these findings, we found an independent significant association between elevated Ang2 and cardiovascular events at all time points in patients on hemodialysis. In patients with CKD, Ang2 is an independent predictor of mortality[55]. In this study, only at 180 min intra-dialysis and post-dialysis an independent association of Ang2 with mortality was found. Presently, we do not know whether the absence of an association of predialysis and 60 min intra-dialysis Ang2 levels is real or is caused by a lack of power.

Our study has several limitations. First, since it is an observational study, conclusions on causality cannot be drawn. Second, our study population was relatively small and was predominantly Caucasian. This limits the generalizability of this study. Strong points are that this is the first study of angiopoietins in relation to markers of inflammation and endothelial function and outcome. These markers were not only measured before, but also during and at the end of HD and were corrected for hemoconcentration.

The high prevalence of chronic inflammation, atherosclerosis and increased mortality risk in dialysis patients renders the study of potential therapeutic targets highly relevant. Pharmacological Ang2 blockade that targets the angiopoietin/Tie2-system might potentially improve outcome in HD patients. Various studies have shown that it is possible to inhibit Ang2-induced Tie2 phosphorylation by antibodies in pre-clinical studies[30,31,56,57]. A peptibody, inhibiting the interaction between the Tie2 receptor and Ang1 and Ang2 was the first to enter a phase III clinical trial demonstrating promising results[58].

The present study shows that Ang2 levels increase significantly during the first hour of HD. The activation of the endothelial layer in hemodialysis is reflected by a disequilibrium in angiopoietins associating with inflammatory and cardiac damage markers. Plasma Ang2 is associated with all-cause mortality and cardiovascular events. The impact on patient outcome underlines the importance of understanding the responsible mechanisms concerning the Ang/Tie2-system. Clarifying this will possibly pave the way for therapeutic intervention studies in hemodialysis.

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Supplementary Table 1. Cox regression analyses for prediction of outcome based on Ang1 levels

	All-cause mortality											
	Ang1 pre-HD*			Ang1 60 min intra-HD*			Ang1 180 min intra-HD*			Ang1 post-HD*		
	HR	95% CI	p	HR	95% CI	p	HR	95% CI	p	HR	95% CI	p
Model 1	1.02	0.56-1.85	0.96	0.75	0.36-1.57	0.44	0.82	0.45-1.50	0.53	0.87	0.40-1.90	0.73
Model 2	1.02	0.54-1.95	0.94	0.84	0.40-1.75	0.64	1.02	0.56-1.86	0.95	0.86	0.40-1.86	0.71
Model 3	1.03	0.54-1.98	0.93	0.82	0.38-1.77	0.62	1.13	0.59-2.13	0.72	0.86	0.40-1.85	0.71
	Cardiovascular events											
Model 1	0.99	0.54-1.85	0.99	1.01	0.49-2.06	0.99	1.33	0.71-2.49	0.37	0.87	0.42-1.83	0.72
Model 2	1.10	0.56-2.02	0.85	1.14	0.55-2.34	0.73	1.42	0.75-2.68	0.28	0.86	0.42-1.76	0.68
Model 3	0.95	0.50-1.78	0.86	0.91	0.43-1.93	0.81	0.99	0.47-2.07	0.97	0.89	0.42-1.87	0.75

*Ang1 was natural-logarithmic (LN) transformed for analyses. Hazard ratios are associated with a 1-unit increase in each covariate. Ang1: angiopoietin-1, CI: Confidence Interval, HR: Hazard Ratio.
Model 1: crude model
Model 2: adjusted for age, sex and dialysis vintage
Model 3: as model 2 + adjusted for diabetes, cardiovascular history and ultrafiltration volume

Supplementary Table 2. Cox regression analyses on the prediction of outcome based on the intradialytic change in Ang1 concentrations

	Δ% Ang1 from pre-HD to post-HD*			Δ% Ang1 from pre-HD to post-HD*		
	All-cause mortality			Cardiovascular events		
	HR	95% CI	p	HR	95% CI	p
Model 1	1.59	0.78-3.25	0.20	1.50	0.73-3.09	0.27
Model 2	1.60	0.78-3.29	0.20	1.38	0.66-2.89	0.39
Model 3	1.56	0.75-3.24	0.23	1.48	0.67-3.26	1.48
Model 4	1.83	0.82-4.10	0.14	1.57	0.65-3.78	0.31

*Concentrations were corrected for the effect of hemoconcentration. 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 dialysis vintage
Model 3: as model 2 + adjusted for diabetes, cardiovascular history and ultrafiltration volume
Model 4: as model 3 + adjusted for predialysis Ang1

Supplementary Table 3. Baseline demographics and characteristics in patients with an intradialytic decrease versus those with an intradialytic increase in Ang2 concentration

	Change from pre- to post-HD		
	Ang2 decrease n = 46	Ang2 increase n = 54	p
Age, years	65 [53-75]	66 [49-75]	0.56
Males, %	67	69	0.90
Dialysis vintage, years	2.2 [0.7-4.0]	1.5 [0.6-3.9]	0.79
Diabetes, %	21	28	0.46
Hypertension, %	79	82	0.69
Predialysis SBP, mmHg	145 ± 27	138 ± 24	0.59
Predialysis DPB, mmHg	82 ± 19	80 ± 15	0.83
Heart rate, BPM	74 ± 14	76 ± 14	0.46
Body mass index, kg/m^2	25 [23-28]	25 [22-28]	0.86
Body surface area, m^2	1.9 ± 0.2	1.9 ± 0.2	0.64
Previous cardiovascular events, %	21	22	0.70
Ultrafiltration rate, ml/kg/h	8.1 ± 2.7	9.0 ± 2.5	0.09
Ultrafiltration volume, ml	2395 ± 745	2729 ± 776	0.04
Hematocrit, %	34 ± 3.3	35 ± 3.7	0.39
Albumin, g/L	40 [38-42]	39 [37-41]	0.65
Calcium, mmol/L	2.3 ± 0.1	2.3 ± 0.2	0.24
Phosphate, mmol/L	1.7 [1.4-2.1]	1.7 [1.3-1.9]	0.40
Kt/V	4.4 [3.9-4.7]	4.1[3.8-4.5]	0.18
Biomarkers			
Angiopoietin-1, ng/ml	3.9 [2-4.5]	2.3 [1.8-4.1]	0.05
Angiopoietin-2, ng/ml	3.6 [2.6-5.5]	1.9 [1.2-3.2]	<0.001
hsCRP, mg/L	6.9 [4-12.8]	6.9 [2.1-14.1]	0.82
Nt-pro-BNP, ng/L	6.3 [2.1-13.3]	3.3 [1.5-5.9]	0.01
Cardiac Troponin T, ng/L	53 [24.5-81.1]	44.7 [28.4-83.3]	0.90
Cardiac Troponin I, ng/L	0.02 [0.01-0.04]	0.02 [0.01-0.03]	0.71
Pentraxin-3, ng/ml	2.6 [1.6-4.2]	2.7 [1.8-4.6]	0.56
Myeloperoxidase, ng/ml	86 [74-101]	81.4 [71.2-97.4]	0.32
IL-6, pg/ml	7.2 [4-9.6]	5.9 [3.5-7.8]	0.34
TNFα, pg/ml	3.6 [2.8-4.6]	3.4 [2.8-4]	0.39
Pro-endothelin, pmol/L	273 [232-331]	280 [220-334]	0.86
Endothelin, ng/ml	34 [18-53]	42 [25-66]	0.09
HbA1C, %	5.5 ± 0.9	5.8 ± 1.1	0.17

Data are presented as mean ± SD in case of normal distribution and as median [interquartile range] in case of skewed distribution. IQR, interquartile range; n, number; HD, hemodialysis; SBP, systolic blood pressure; DBP, diastolic blood pressure; BPM, beats per minute; hsCRP, high-sensitive CRP; Nt-pro-BNP, N-terminal pro-Brain Natriuretic Peptide; IL-6, interleukin-6; TNF-α, tumor necrosis factor α; HbA1C, glycosylated hemoglobin. P for difference was tested by the Mann-Whitney U test for continuous variables or Chi-square test for binary variables.