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DISSERTATION

Circulating Angiopoietin-2 level is independently associated with all-cause mortality in male end-stage kidney disease patients on hemodialysis

Angiopoietin-2 ist ein unabhängiger Risikofaktor der Gesamtmortalität bei männlichen Hämodialysepatienten

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Table of contents

List of tables

List of figures

List of abbreviations

Ang-2	angiopoietin-2
anti-GBM GN	anti-glomerular basement membrane glomerulonephritis
AUC	area under the roc curve
BMI	body mass index
CHD	coronary heart disease
CKD	chronic kidney disease
CRP	c-reactive protein
CVD	cardiovascular disease
DBP	diastolic blood pressure
EC	endothelial cell
ELISA	enzyme-linked immunosorbent assay
ESRD	end-stage renal disease
Hb	hemoglobin
HD	hemodialysis
HDL	high-density lipoprotein
HR	hazard ratio
HRP	horseradish peroxidase
iPTH	intact parathyroid hormone
IQR	interquartile ranges
LDL	low-density lipoprotein
pmp	per million population
RAAS	renin-angiotensin-aldosterone system
ROC	receiver operating characteristic
RRT	renal replacement therapy
RTR	renal transplant recipient
SBP	systolic blood pressure
sCr	serum creatinine
TMB	tetramethylbenzidine
VEGF	vascular endothelial growth factor
WPB	Weibel-Palade body
95% CI	95% confidence interval

Abstract

Abstract (English)

End-stage renal disease (ESRD) patients receiving hemodialysis (HD) continue to have a low quality of life and face severe mortality. Biomarkers involved in deterioration and predicting the clinical outcome in ESRD patients on HD are needed. Abnormal angiogenesis has been noted in kidney diseases, characterized by a disruption of balance in the expression of pro-angiogenic and anti-angiogenic factors. The most dominant pro-angiogenic factor, vascular endothelial growth factor, has shown a critical effect in the pathogenesis of kidney diseases in past studies. Moreover, Angiopoietin-2 (Ang-2), as one of the most well-characterized vascular growth factors, its circulating level is increased in HD patients and has been reported to have a prognostic significance for mortality in patients with chronic kidney disease. Together with previous evidence of sexual dimorphism in endothelial cell-derived growth factors, this study evaluated the prognostic value of Ang-2 in HD patients and its sexual difference. Prevalent HD patients were recruited and followed up for five years for all-cause mortality. Circulating Ang-2 concentrations were assessed by validated ELISA. Survival analysis was performed using Kaplan-Meier analysis and Cox proportional hazards regression analysis. There were 157 deaths among 313 participants during the follow-up. The median level of Ang-2 was 91.20 pmol/l (interquartile range 63.35, 140.65 pmol/l). Ang-2 concentration was significantly lower in survivors than in non-survivors ($p<0.0001$), and this finding was significant in male patients ($p<0.0001$), but not in female patients ($p=0.249$). Male HD patients with lower Ang-2 levels (<111.0 pmol/l) were associated with a decrease in all-cause mortality ($p<0.0001$). In multivariate Cox regression analyses, after adjusting for factors known to be associated with Ang-2 and mortality, elevated Ang-2 was independently associated with an increased risk of all-cause mortality in male HD patients (HR, 3.294, 95% CI, 1.768-6.138, $p=0.0002$ for males, HR, 1.084, 95% CI, 0.476-2.467, $p=0.847$ for females). In conclusion, this study revealed a significant sex difference in the association between serum Ang-2 levels and all-cause mortality in prevalent HD patients.

The risk of death was increased in male patients with elevated Ang-2 levels. Furthermore, Ang-2 may not only be a biomarker for predicting the outcome but is likely a potent vascular hormone that contributes to the pathophysiology of vascular injury in kidney disease. Present study after independent validation may inspire the development of Ang-2 antagonists to improve outcomes in male ESRD patients.

Zusammenfassung (Deutsch)

Patienten mit terminaler Niereninsuffizienz, die eine Hämodialyse (HD) erhalten, haben nach wie vor eine geringe Lebensqualität und eine hohe Mortalität. Es werden Biomarker benötigt, die an der Verschlechterung der Lebensqualität beteiligt sind und das klinische Ergebnis bei Patienten mit terminaler Niereninsuffizienz vorhersagen. Bei Nierenerkrankungen wurde eine abnorme Angiogenese festgestellt, die durch eine ausgewogene Störung der Expression von pro- und anti-angiogenen Faktoren gekennzeichnet ist. Angiopoietin-2 (Ang-2), einer der am besten charakterisierten vaskulären Wachstumsfaktoren, ist im Blutkreislauf von HD-Patienten in erhöhter Konzentration vorhanden und hat Berichten zufolge eine prognostische Bedeutung für die Mortalität von Patienten mit chronischen Nierenerkrankungen. Zusammen mit früheren Hinweisen auf Geschlechtsunterschiede bei den aus Endothelzellen hergestellten Wachstumsfaktoren untersuchte diese Studie den prognostischen Wert von Ang-2 bei HD-Patienten und dessen Geschlechtsunterschiede. Prävalente HD-Patienten wurden rekrutiert und fünf Jahre lang nachbeobachtet, um die Gesamtmortalität zu untersuchen. Zu Beginn wurden demografische und medizinische Daten erhoben, und die zirkulierenden Ang-2-Konzentrationen wurden mit einem validierten ELISA bestimmt. Während der Nachbeobachtungszeit starben 157 der 313 Teilnehmer. Der Medianwert von Ang-2 betrug 91,20 pmol/l (Interquartilsbereich 63,35 bis 140,65 pmol/l). Die Ang-2-Konzentration war bei den Überlebenden signifikant niedriger als bei den Nicht-Überlebenden ($p<0,0001$), und dieser Befund war signifikant bei männlichen Patienten ($p<0,0001$), aber nicht bei weiblichen Patienten ($p=0,249$). Männliche HD-Patienten mit einem niedrigeren Ang-2-Spiegel (<111,0 pmol/l) waren mit einem Rückgang der Gesamtmortalität verbunden ($p<0,0001$). In multivariaten Cox-Regressionsanalysen, nach Anpassung an Faktoren, von denen bekannt ist, dass sie mit Ang-2 und der Sterblichkeit in Zusammenhang stehen, war ein erhöhter Ang-2-Wert bei männlichen HD-Patienten unabhängig mit einem erhöhten Risiko für die Gesamtsterblichkeit verbunden (HR, 3,294, 95% CI, 1,768-6,138, $p=0,0002$ für Männer, HR, 1,084, 95% CI, 0,476-2,467, $p=0,847$ für Frauen). In dieser Studie wurde ein signifikanter geschlechtsspezifischer

Unterschied im Zusammenhang zwischen den Ang-2-Serumspiegeln und der Gesamtmortalität bei HD-Patienten festgestellt. Das Sterberisiko war bei männlichen Patienten mit erhöhtem Ang-2-Spiegel erhöht. Außerdem könnte Ang-2 nicht nur ein Biomarker für das Ergebnis sein, sondern wahrscheinlich auch ein starkes Gefäßhormon, das zur Pathophysiologie der Gefäßschäden bei Nierenerkrankungen beiträgt. Die vorliegende Studie könnte nach unabhängiger Validierung die Entwicklung von Ang-2-Antagonisten anregen, um die Ergebnisse bei Patienten mit terminaler Niereninsuffizienz zu verbessern.

1 Introduction

1.1 Angiopoietin-2 in vascular physiology and pathophysiology

Angiogenesis is involved in many physiological situations and pathological situations with endothelial dysfunction in response to tissue damage or deficiencies of oxygen and nutrients (1, 2). A common pathological situation is a tumor-induced angiogenesis, where new vascular networks are rapidly developed to support the high proliferative rate of cancer cells (3). Tumoral microvascular density is an independent predictor of metastasis and overall survival in cancer patients (4). In addition, angiogenesis also has wide-ranging effects on ischemic diseases and inflammatory disorders (1). Angiogenesis is induced by an imbalance between pro-angiogenic and anti-angiogenic factors (5), and angiopoietins are regarded as one of the most important angiogenic factors (4).

Angiopoietin-2 (Ang-2) is one of the most well-characterized vascular growth factors belonging to the angiopoietin/Tie signaling system. All molecules of this system are signaling toward the vascular-specific receptor tyrosine kinase pathway that plays a fundamental role in angiogenesis (6). Ang-2, a 496 amino acid long protein with approximately 60% amino acid homology to angiopoietin-1 (Ang-1), was shortly identified after Ang-1 by homology screening (7). Ang-1 and Ang-2 regulate vascular remodeling, maturation, and stabilization via counteracting actions (8). Ang-1 is a potent agonist of the Tie2 receptor, combines and phosphorylates Tie2 receptor, which then signals anti-inflammatory to the endothelium, thus, regulating the survival of endothelial cells (ECs) and therefore stabilizing vascular structure. Ang-2 binds to the Tie2 with the same binding affinity as Ang-1. However, Ang-2 acts as a natural antagonist of Ang-1 and competes with Ang-1 for combining with Tie2, thus, leading to degradation of the basal lamina and vascular instability (8-10).

Ang-2 is mainly generated by ECs and deposited in endothelial Weibel-Palade bodies (WPBs) (11), acts in an autocrine manner, and its expression is highly regulated (6). The Ang-2 expression may be upregulated in various conditions, such as inflammation (12), hypoxia (13), and cancer (14). Increased Ang-2 level has been published in patients with

endothelial dysfunction and vascular inflammation, such as diabetes mellitus (15), chronic kidney diseases (CKD) (16), cardiovascular diseases (CVD) (17), systemic lupus erythematosus (18) and systemic inflammatory response syndrome (19, 20).

1.2 Role of Angiopoietin-2 in kidney

Ang-2 is widely expressed during kidney development and plays considerable roles in the maturation of glomeruli and renal blood vessels. However, it is significantly downregulated in the adult kidney, so Ang-2 is very low in normal mature glomeruli (21, 22). In chronic kidney injury with nephron reduction, significant remodeling and proliferation of peritubular capillaries in the renal cortex were accompanied by increased renal vascular endothelial growth factor (VEGF) protein levels (23). Furthermore, past studies discovered that regression of glomerular endothelial cells is associated with apoptosis, which may affect the progression of glomerulosclerosis and has been reported as a feature of experimental models of glomerular disease, including remnant kidneys (22, 24). Thus, Ang-2, as a crucial regulator of glomerular vascular remodeling and ECs stabilization, has been implicated in the pathological process of glomerular diseases.

It has been shown that Ang-2 expression is significantly increased in glomeruli of animal models of several kidney diseases. Yuan HT et al. (22) reported that glomerular capillaries loss was associated with upregulated Ang-2 during anti-glomerular basement membrane glomerulonephritis (anti-GBM GN) in a mice model. In acute anti-Thy1.1 glomerulonephritis, there was a higher expression of Ang-2 within the glomeruli with the disease than in controls, and it played a pivotal role in disrupting endothelial homeostasis (25). Lu YH et al. (26) found that increased Ang-2 in plasma and glomeruli may mediate the permeability of proteins through the glomerular filtration barrier in a daunorubicin-induced progressive glomerulosclerosis rat model. In addition, local expression of Ang-2 may promote the progression of glomerulosclerosis by upregulating the expression of components of the extracellular matrix. Moreover, the expression of Ang-2 was also increased in the animal model of diabetic nephropathy (27, 28).

In clinical studies, increased blood Ang-2 level was discovered in CKD patients and

was associated with advanced pathological features and deterioration of renal function. David S et al. (29) found that plasma Ang-2 level steadily increased in parallel to the progression of CKD, and there is a significant negative correlation between Ang-2 level and GFR in patients with stages 1-4 CKD. Over a four-year observation period of patients with stages 4-5 CKD, elevated circulating Ang-2 levels strongly predicted long-term mortality, and this predictive value is independent of conduit arterial stiffness or vascular calcification (30). Later, Chang FC et al. (31) reported the independent association of circulating Ang-2 level with albuminuria and micro-inflammation, both of which are associated with increased risk of CVD and mortality in CKD patients. Besides, Tsai YC et al. (32) evaluated the association of Ang-2 level with adverse renal outcomes in patients with CKD during a three-year observation period. They reported that Ang-2 level was associated with a composite renal outcome (initiation of dialysis or creatinine doubling) even after adjusting for baseline renal function and relevant risk factors. Furthermore, high Ang-2 levels were positively associated with structural cardiac abnormalities in patients with stages 3-5 CKD, implying that Ang-2 might participate in cardiovascular burdens (16).

1.3 Dialysis and Angiopoietin-2

The incidence of end-stage renal disease (ESRD) is increasing globally. According to the latest annual report 2019 from European Renal Association (https://www.era-online.org/registry/AnnRep2019.pdf), 89,579 people out of a population of 680 million started renal replacement therapy (RRT) for ESRD in 2019, resulting in an overall unadjusted incidence of 132 per million population (pmp). On 31 December 2019, 607, 320 patients were receiving RRT for kidney failure, corresponding to an overall unadjusted prevalence of 893 pmp. Of all patients, 61% of the patients were male, 55% were under 65 years old, and the top four known causes of primary renal diseases were glomerulonephritis/sclerosis (18%), diabetes mellitus (15%), miscellaneous (14%) and hypertension (10%). When RRT was initiated, hemodialysis (HD) was the primary treatment modality (84%). In prevalent patients, 58% of patients were on HD, 5% were

on peritoneal dialysis, and 37% had kidney transplants. As a result, patients with ESRD continue to suffer a low quality of life and face severe mortality. For patients who initiated RRT during 2010-2014, the unadjusted five-year probability of survival was 51.9% (95% CI, 51.6-52.1). For patients who started RRT with dialysis during this period, the unadjusted five-year probability of survival was 42.3% (95% CI 42.1-42.6).

The major cause of death was cardiovascular disease in ESRD patients on HD treatment (33). At the same time, conventional risk factors, like diabetes, dyslipidemia, hypertension, aging, etc., are insufficient to predict the higher risk of cardiovascular disease in ESRD patients on HD patients than in the general population or renal transplant recipients (RTRs) (34, 35). There is growing evidence of increased circulating Ang-2 levels in patients treated with dialysis (30, 36, 37), and its prognostic significance for cardiovascular disease has been reported only in children on chronic dialysis (37). Interestingly, David S et al. (36) found that kidney transplantation normalized circulating Ang-2 levels after three months. The mechanism of this discrepancy of Ang-2 levels in different RRT is unclear so far. It is believed that dialysis is related to a critical imbalance of angiopoietins, which may reflect a perpetual destructive activation of the endothelium (36). In addition, Ang-2 may act as a mediator of micro-inflammation in the HD population (38). Furthermore, prior studies indicated that Ang-2 is involved in cardiovascular disorders, which could account for accelerated atherosclerosis in ESRD patients on HD; however, it but did not correlate with atherosclerosis in RTRs (36, 37). So far, the association of Ang-2 levels with all-cause mortality in adults on HD treatment has not been investigated yet.

2 Methods

2.1 Study Population

To determine associations of Ang-2 with all-cause mortality in hemodialysis patients, a prospective cohort study was conducted. We recruited a total of prevalent 340 patients on hemodialysis from different dialysis centers associated with our inpatient facility at the Campus Charité Mitte (KfH Dialysezentrum-Neukölln, Berlin, Germany, and KfH Dialysezentrum-Moabit, Berlin, Germany). The patients were followed up for all-cause mortality for five years. All patients underwent dialysis at least three times a week for four-five hours each time, utilizing standard bicarbonate dialysis and biocompatible membranes. Dialysate flow rates were 500 mL/min, and blood flow rates were 250-300 mL/min. All patients had a functioning permanent vascular access. Patients were excluded from the study if they were pregnant or had any malignancy or active infections, were unwilling or unable to participate, or were unable to provide written informed consent. In addition, patients who had a kidney transplant during the period of follow-up were censored at the point of transplant. The local ethics committee authorized the study and informed consents were acquired from all patients before enrolling in this study.

2.2 Data Collection

Baseline demographics and medical data were obtained, i.e., age, sex, weight, height, body mass index (BMI, weight in kilograms divided by height in meters squared), underlying kidney disease, dialysis vintage, systolic and diastolic blood pressure (SBP and DBP), comorbidity (presence of diabetes, hypertension, or coronary heart diseases), smoking status, medication [use of renin-angiotensin-aldosterone system (RAAS) inhibitors, beta-blockers, calcium channel blockers, and erythropoietin]. Blood samples were collected from patients before the HD session at the study entrance. The following parameters, hemoglobin (Hb), ferritin, transferrin, serum albumin, low-density lipoprotein (LDL), high-density lipoprotein (HDL), serum cholesterol, serum triglycerides, C-reactive protein (CRP), serum urea, serum creatinine (sCr), serum potassium, serum calcium,

serum phosphate and intact parathyroid hormone (iPTH) were assessed in the clinical laboratory using standardized methods. Kt/V (K, dialyzer clearance of urea; t, the dialysis time; V, the volume of distribution of urea, approximately equal to the total body water of the patient), a number used to quantify dialysis treatment adequacy, was calculated.

2.3 Measurement of Angiopoietin-2 concentrations

Ang-2 concentrations were measured using *Human Angiopoietin-2 ELISA* kit (cat. no. BI-ANG2) from Biomedica (Vienna, Austria) with manufactural instruction (https://www.bmgrp.com/wp-content/uploads/2019/11/BI-ANG2-Angiopoietin-2-ELISA-Validation-Data-191128.pdf). The detection limit of the ELISA kit was 3.7 pmol/l. The average intra- and inter-assay coefficients of variation were <8% and <6%, respectively. All reagents, standard dilutions, control, and samples were prepped following the manufactural instruction. The well positions of standards/controls/samples were marked on a protocol sheet. First, previously prepared standards/controls/samples were pipetted into the wells of pre-coated microplate. The plate was then sealed airtight and incubated for two hours at room temperature on a 500-rpm horizontal orbital shaker. Second, the content of each well was discarded, and each well was aspirated and washed five times with wash buffer. After the last wash, the plate was inverted and tapped on absorbent paper to remove excess liquid. Third, 100 µL of the detection antibody solution was pipetted into each well and then incubated with the plate for two hours with constant gentle shaking (~500 rpm). In this step, the target antigen in the standards/controls/samples is attached to the pre-coated antibody in the well and then built as a sandwich with the antibody. Then the washing (second) step was repeated. In the washing step, all non-specific and non-binding substances were cleared. Fourth, 100µl conjugate (Streptavidin-HRP) was added to each well, and the plate was then sealed and incubated for two hours on the shaker. In this process, the conjugate reacted with the biotinylated antibody. Then the washing (second) step was repeated. Fifth, 100µL tetramethylbenzidine (TMB) substrate solution was pipetted into each well. The color change of the substrate catalyzed by the enzyme is proportional to the quantity of the

target protein existing in the sample. After incubating for 30 minutes at room temperature in the dark, 100μL stop solution was pipetted into each well and thoroughly mixed. Last, the optical density of each well was measured within 30 minutes on a standard microplate reader, which was set to 450 nm, to determine concentrations of the target protein in samples from a dose-response curve.

2.4 Statistical analysis

Baseline demographics and medical data are presented as medians (interquartile ranges, IQR) or numbers (percentage, %). Comparisons between groups were assessed by the Mann-Whitney U test for continuous variables and by χ^2 test for categorical variables. The optimal cut-off value of Ang-2 concentration for all-cause mortality was calculated based on the receiver operating characteristic (ROC) analysis, mapping the sensitivity versus 1-specificity (39, 40). In continuation, the calculation was done by the Youden index (J) method (41), and the optimal cut-point was defined as the point at which the Youden function is maximized, i.e., the difference between the true positive rate and the false positive rate among all possible cut-point values (42, 43). To avoid potential overoptimism (bias) in the estimated hazard ratios, we not only use the cut-off value based on the ROC analysis in all participants, male and female, but also median values of Ang-2 in multivariate cox regression analysis. Time-to-event analysis was estimated through Kaplan-Meier analysis with optimal cut-point, followed by a log-rank test to assess differences. Multivariable Cox regression analysis was conducted in three models (A-C) with different ROC-derived cut-off values (ROC-based cut-off for the entire patients, ROC-based cut-off for male patients, and ROC-based cut-off for female patients) and the median value of Ang-2 level of the entire patients. Model A was adjusted for demographics data; age, comorbidities (diabetes, hypertension, and coronary heart diseases), and smoking status. Model B was adjusted for medical data; dialysis vintage, sCr, Hb, CRP, serum albumin, ferritin, transferrin, iPTH, serum calcium, serum phosphorus, LDL, Kt/V. Model C was adjusted for all model A and B risk factors, plus ultrafiltration volume. P value < 0.05 was regarded as statistically significant. Statistical analyses were performed using

SPSS version 25.0 (Chicago, IL, USA), and figures were created using GraphPad Prism version 8.0 (California, USA).

3 Results

3.1 Participants & descriptive data in the entire cohort

Finally, 313 patients were included in the statistical analysis after 27 patients were excluded because of blood sample limitation for Ang-2 determinations. In this cohort, the cause of CKD is comprised of hypertensive nephropathy (34.2%), diabetic nephropathy (31.0%), glomerulonephritis (8.5%), polycystic kidney disease (2.9%), and other or unknown reasons (24.0%). During a five-year follow-up, 157 patients (50.2%, 102 males and 55 females) died, and 41 patients (13.1%) had kidney transplants.

Table 1 presents the baseline demographics and medical data of patients by the median concentration of Ang-2 (91.2 pmol/L). The median age of this cohort was 66 years old, the median dialysis vintage (time since dialysis started) was 243 days, and the median dialysis dose (Kt/V) was 1.2. Comparisons between patients whose Ang-2 concentration was below and above the median show that patients with lower baseline Ang-2 concentrations were on average younger, had shorter dialysis vintage, lower levels of ferritin, CRP, sCr, and serum potassium, but higher levels of transferrin and LDL compared to patients with Ang-2 concentrations above the median. Moreover, in the higher Ang-2 level group, fewer patients took beta-blockers than in the lower Ang-2 group, but more had erythropoietin treatment than in the lower Ang-2 group.

Table 1. Baseline demographics and medical data of hemodialysis patients by median concentration of Ang-2 (91.2 pmol/L).

Characteristic	All	Ang-2 ≤91.2 pmol/l	Ang-2 >91.2 pmol/l
N	313	158	155
Age, years	66 (56, 75)	65 (55, 72) *	69 (58, 77)
Diabetes mellitus, %	117 (37.4%)	59 (37.3%)	58 (37.4%)
Hypertension, %	246 (78.6%)	120 (75.9%)	126 (81.3%)
CHD, %	148 (47.3%)	71 (44.9%)	77 (49.7%)
Smoker, %	97 (31.0%)	55 (34.8%)	42 (27.1%)
Body mass index, kg/m^2	24.5 (22.0, 27.6)	24.6 (22.0, 28.4)	24.5 (22.0, 27.0)
Dialysis vintage, days	243 (31, 1172)	152 (31, 1142) *	366 (64, 1186)
Medication, n (%)			
RAAS inhibitors	82 (26.2%)	45 (28.5%)	37 (23.9%)
Beta-blockers	186 (59.4%)	103 (65.2%) *	83 (53.5%)
Calcium channel blockers	98 (31.3%)	56 (35.4%)	42 (27.1%)
Erythropoietin	158 (50.5%)	71 (44.9%) *	87 (56.1%)
Hemoglobin, g/dL	10.2 (9.1, 11.5)	10.1 (8.8, 11.3)	10.3 (9.2, 11.7)
Ferritin, ng/mL	517 (244, 1074)	446 (194, 856) **	681 (308, 1253)
Transferrin, mg/dL	138 (107, 173)	150 (122, 176) **	127 (102, 164)
Serum albumin, g/dL	3.3 (2.9, 3.7)	3.4 (3.0, 3.7)	3.1 (2.8, 3.7)
C-reactive protein, mg/dL	2.6 (1.0, 5.0)	2.1 (0.7, 4.0) ***	3.1 (1.2, 7.3)
Total cholesterol, mg/dL	151 (127, 187)	161 (134,197) *	145 (120, 178)
Triglycerides, mg/dL	159 (110, 248)	167 (117, 270) *	137 (101, 212)
HDL, mg/dL	40 (32, 50)	41 (34, 51)	39 (31, 50)
LDL, mg/dL	93 (72, 120)	102 (79, 128) **	86 (67, 108)
Urea, mg/dL	196 (147, 269)	208 (152, 283) *	190 (135, 242)
Serum creatinine, mg/dL	6.7 (4.3, 8.4)	5.7 (3.8, 7.9) **	7.1 (4.8, 8.7)
Serum potassium, mmol/L	4.7 (4.1, 5.3)	4.5 (4.0, 5.1) ***	4.9 (4.3, 5.5)
Serum calcium, mmol/L	2.2 (2.1, 2.4)	2.2 (2.1, 2.4)	2.3 (2.1, 2.4)
Serum phosphorus, mmol/L	1.61 (1.20, 2.10)	1.50 (1.12, 2.01)	1.70 (1.23, 2.10)
iPTH, ng/L	50.2 (19.0, 129.6)	53.5 (22.0, 149.8)	44.8 (15.6, 118.6)
Dialysis dose, Kt/V	1.19 (1.07, 1.33)	1.17 (1.06, 1.32)	1.21 (1.08, 1.36)
Angiopoietin 2, pmol/L	91.20 (63.35, 140.65)	63.55 (46.55, 76.73) ***	141.20 (114.00, 173.30)

Continuous variables are given as medians and interquartile range. Between groups, comparisons were made using Mann-Whitney U test for continuous variables and by χ^2 test for categorical variables. *p<0.05; **p<0.01; ***p<0.001, comparison between patients Ang-2 ≤91.2 pmol/l and Ang-2 >91.2 pmol/l. Body mass index was calculated as weight in kilograms divided by height in meters squared. This table was modified from our published paper (44).

3.2 Serum Angiopoietin-2 and all-cause mortality in the entire cohort

3.2.1 Comparison between survivors and non-survivors

In this cohort, the median serum Ang-2 level was 91.2 pmol/L (IQR, 63.4 to 140.7 pmol/L) in this cohort. Ang-2 concentration was significantly lower in survivors than non-survivors during a five-year follow-up [78.6 (53.3, 117.2) pmol/L vs. 112.5 (72.0, 153.8) pmol/L, p<0.0001] (figure 1).

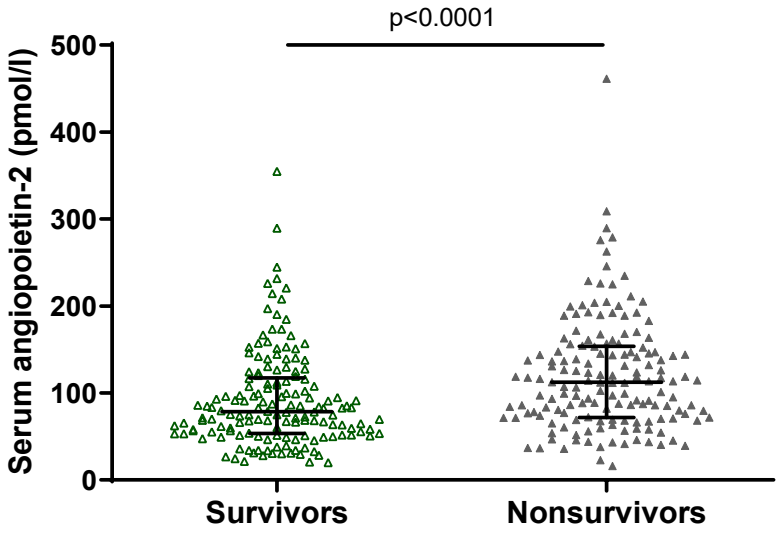

Figure 1. Plots of serum Ang-2 concentrations in survivors and non-survivors. Line was presented at median with interquartile range, 78.6 (53.3, 117.2) pmol/L in survivors and 112.5 (72.0, 153.8) pmol/L in non-survivors. Ang-2 concentration was significantly lower in survivors than non-survivors during a five-year follow-up (p<0.0001). This figure is modified from our published paper (44).

3.2.2 ROC analysis

The optimal cut-off value of baseline Ang-2 for predicting all-cause mortality was 111.0 pmol/L in all participants based on the ROC analysis (AUC=0.65, p<0.0001) (Figure 2).

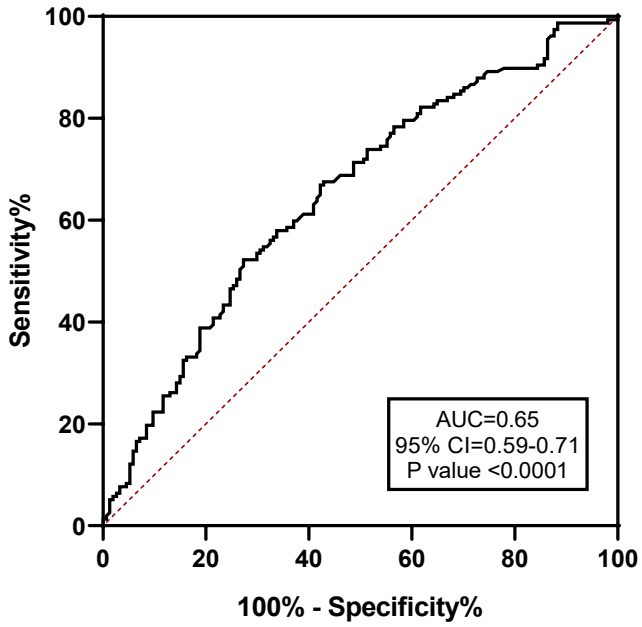

Figure 2. Receiver operating characteristic (ROC) curves for all-cause mortality. The optimal cut-off value for baseline serum angiopoietin-2 to predict all-cause mortality was 111.0 pmol/L (AUC=0.65, 95%CI 0.59-0.71, p<0.0001). This figure is modified from our published paper (44).

3.2.3 Kaplan-Meier survival analysis

In Kaplan-Meier survival analysis, patients with lower Ang-2 level (<111.0 pmol/L) showed a significantly higher survival rate than those with higher Ang-2 level (≥111.0 pmol/L) (log-rank test, p<0.0001) (Figure 3).

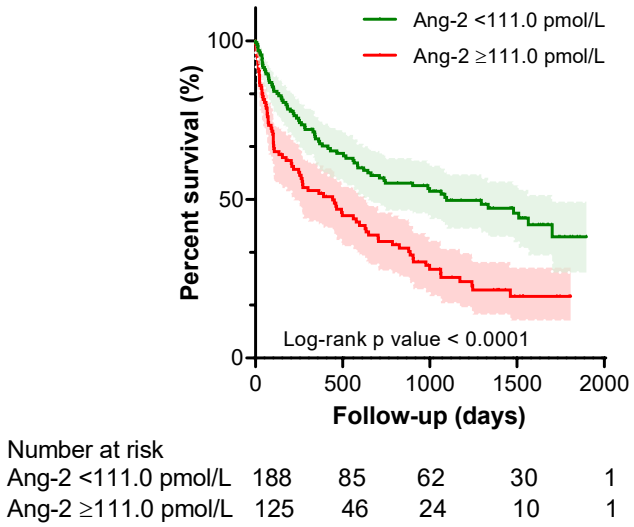

Figure 3. Kaplan-Meier survival curve for patients below and above optimal predictive value of angiopoietin-2 (111.0 pmol/L). The shaded area represents the 95% confidence interval for the curve. This figure is modified from our published paper (44).

3.2.4 Cox regression analysis

Binary Ang-2 was classified in accordance with the ROC-derived cut-off value of the entire participants (111.0 pmol/L), and the median of the entire participants (91.2 pmol/L). Univariate Cox regression analyses revealed that patients with either increased Ang-2 or in the binary upper half of the Ang-2 group had a significantly higher all-cause mortality risk. Then, multivariable Cox regression analyses were conducted in A-C models, model A was adjusted for age, comorbidities (diabetes, hypertension, and coronary heart diseases), and smoking status. Model B was adjusted for dialysis vintage, serum creatinine, hemoglobin, C-reactive protein, serum albumin, ferritin, transferrin, iPTH, serum calcium, serum phosphorus, LDL, Kt/V. Finally, model C was adjusted for all model A and B risk factors, and ultrafiltration volume. Ang-2 concentrations presented a consistent and positive association with all-cause mortality in all models (Table 2).

Table 2. Multiple cox regression models analyzing serum angiopoietin-2 as a predictor of all-cause mortality.

	HR (95% CI)	P value
Univariate Cox regression		
Continuous angiopoietin-2	1.002 (1.001-1.004)	0.005
Binary angiopoietin-2 [a]	1.934 (1.412-2.649)	<0.0001
Binary angiopoietin-2 [b]	1.697 (1.231-2.338)	0.001
Multivariable Cox regression [a]		
Model A	1.756 (1.274-2.419)	0.001
Model B	2.613 (1.698-4.023)	<0.0001
Model C	2.245 (1.443-3.493)	0.0003
Multivariable Cox regression [b]		
Model A	1.609 (1.156-2.239)	0.005
Model B	2.108 (1.358-3.271)	0.001
Model C	1.741 (1.110-2.732)	0.016

[a] Binary Ang-2 was divided by ROC-derived cut-off value of the entire participants (111.0 pmol/l), [b] Binary Ang-2 was divided by median of the entire participants (91.2 pmol/l). Model A was adjusted for age, comorbidities (diabetes, hypertension, and coronary heart diseases), and smoking status. Model B was adjusted for dialysis vintage, serum creatinine, hemoglobin, C-reactive protein, serum albumin, ferritin, transferrin, iPTH, serum calcium, serum phosphorus, LDL, Kt/V. Model C was adjusted for all risk factors in model A and B, plus ultrafiltration volume. This table was modified from our published paper (44).

3.3 Sex-related differences

3.3.1 Participants & descriptive data

Table 3 presents all patients' baseline demographics and medical data by sex. Male patients comprised 65.8% of the cohort (n=206). When comparing male and female patients, male patients appeared to be more often diabetic and smoker. They had lower HDL, total cholesterol levels, and dialysis doses but higher CRP and sCr levels than females.

Table 3. Baseline demographics and medical data of hemodialysis patients by sex.

Characteristic	All	Male	Female
N	313	206	107
Age, years	66 (56, 75)	66 (56, 74)	67 (57, 76)
Diabetes mellitus, %	117 (37.4%)	66 (32.0%) ##	51 (47.7%)
Hypertension, %	246 (78.6%)	160 (77.7%)	86 (80.4%)
CHD, %	148 (47.3%)	99 (48.1%)	49 (45.8%)
Smoker, %	97 (31.0%)	75 (36.4%) ##	22 (20.6%)
Body mass index, kg/m^2	24.5 (22.0, 27.6)	24.9 (22.3, 27.4)	24.1 (21.6, 28.5)
Dialysis vintage, days	243 (31, 1172)	271 (31, 1347)	227 (31, 919)
Medication, n (%)			
RAAS inhibitors	82 (26.2%)	54 (26.2%)	28 (26.2%)
Beta-blockers	186 (59.4%)	116 (56.3%)	70 (65.4%)
Calcium channel blockers	98 (31.3%)	65 (31.6%)	33 (30.8%)
Erythropoietin	158 (50.5%)	104 (50.5%)	54 (50.5%)
Hemoglobin, g/dL	10.2 (9.1, 11.5)	10.1 (9.0, 11.2)	10.2 (9.2, 11.6)
Ferritin, ng/mL	517 (244, 1074)	513 (239, 1151)	520 (243, 914)
Transferrin, mg/dL	138 (107, 173)	138 (104, 172)	142 (116, 177)
Serum albumin, g/dL	3.3 (2.9, 3.7)	3.3 (2.9, 3.7)	3.2 (2.8, 3.6)
C-reactive protein, mg/dL	2.6 (1.0, 5.0)	3.1 (1.2, 6.0) ###	1.4 (0.7, 3.3)
Total cholesterol, mg/dL	151 (127, 187)	147 (120, 182) ##	166 (134, 204)
Triglycerides, mg/dL	159 (110, 248)	159 (106, 248)	163 (112, 248)
HDL, mg/dL	40 (32, 50)	36 (31, 46) ###	44 (36, 57)
LDL, mg/dL	93 (72, 120)	93 (70, 114)	103 (76, 129)
Urea, mg/dL	196 (147, 269)	205 (146, 280)	183 (151, 242)
Serum creatinine, mg/dL	6.7 (4.3, 8.4)	6.9 (4.6, 8.7) ##	5.7 (3.8, 7.5)
Serum potassium, mmol/L	4.7 (4.1, 5.3)	4.8 (4.1, 5.3)	4.6 (4.0, 5.2)
Serum calcium, mmol/L	2.2 (2.1, 2.4)	2.3 (2.1, 2.4)	2.2 (2.1, 2.4)
Serum phosphorus, mmol/L	1.61 (1.20, 2.10)	1.63 (1.21, 2.10)	1.59 (1.17, 2.01)
iPTH, ng/L	50.2 (19.0, 129.6)	47.7 (19.0, 131.9)	52.8 (18.9, 127.4)
Dialysis dose, Kt/V	1.19 (1.07, 1.33)	1.16 (1.06, 1.31) #	1.24 (1.11, 1.44)
Angiopoietin 2, pmol/L	91.20 (63.35, 140.65)	94.30 (64.48, 143.78)	86.30 (59.30, 127.70)

Continuous variables are given as medians and interquartile range. Between groups, comparisons were made using Mann-Whitney U test for continuous variables and by χ^2 test for categorical variables. #p<0.05; ##p<0.01; ###p<0.001, comparison between male patients and female patients. Body mass index was calculated as weight in kilograms divided by height in meters squared. This table was modified from our published paper (44).

3.3.2 Comparison between survivors and non-survivors

Serum Ang-2 concentration was significantly lower in survivors than non-survivors of males [78.40 (51.45, 118.50) vs. 119.80 (76.98, 161.60) pmol/L, p< 0.0001]. However, there were no significant differences when comparing survivors and non-survivors of females [80.20 (59.00, 117.70) vs. 91.20 (67.20, 142.9) pmol/L, p=0.249] (Figure 4).

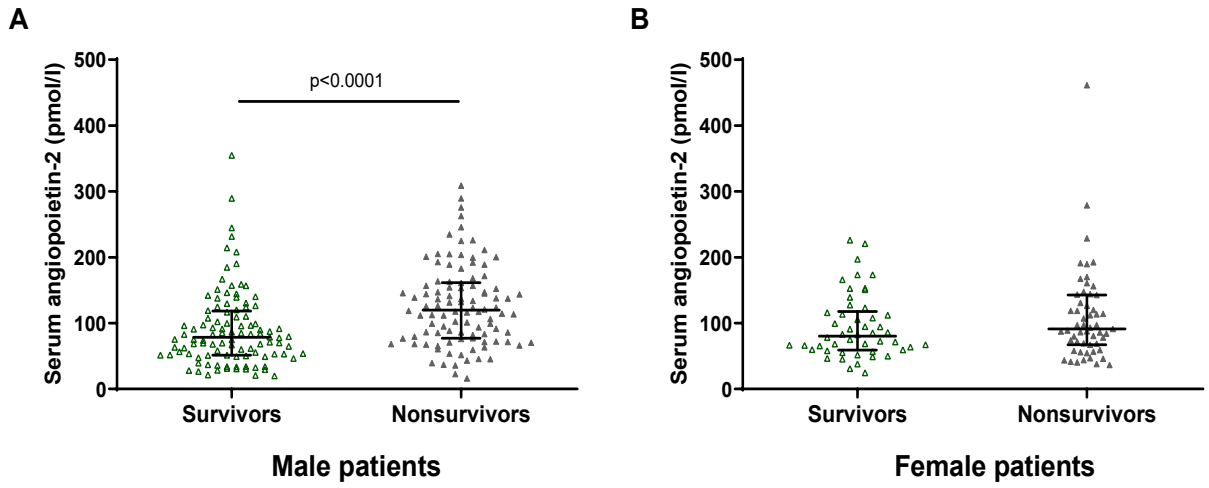

Figure 4. (A) Plots of serum Ang-2 concentrations in male survivors and non-survivors. Line was presented at median with interquartile range, 78.4 (51.5, 118.5) pmol/L in survivors and 119.8 (77.0, 161.6) pmol/L in non-survivors. Ang-2 concentration was significantly lower in survivors than non-survivors during a five-year follow-up (p<0.0001). (B) Plots of serum Ang-2 concentrations in female survivors and non-survivors. Line was presented at median with interquartile range, 80.2 (59.0, 117.7) pmol/L in survivors and 91.2 (67.2, 142.9) pmol/L in non-survivors. No statistical significance between survivors and non-survivors during a five-year follow-up (p=0.249). This figure is modified from our published paper (44).

3.3.3 ROC analysis

The optimal cut-off value was 99.1 pmol/L in male patients (AUC=0.69, p<0.0001), and 85.9 pmol/L in female patients (AUC=0.57, p=0.249) (Figure 5).

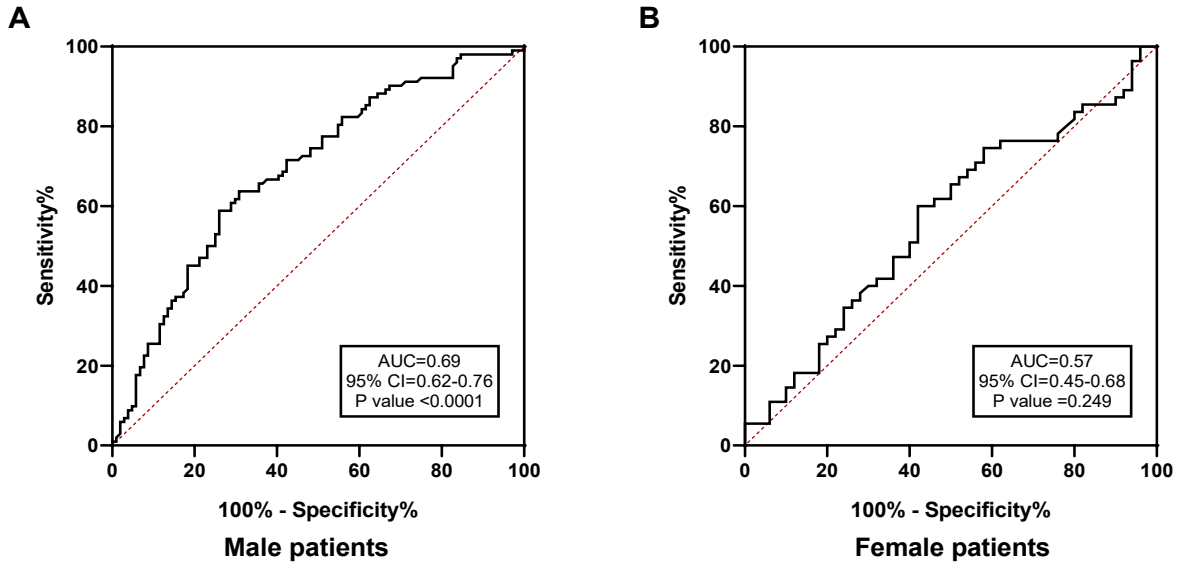

Figure 5. (A) Receiver operating characteristic (ROC) curves for all-cause mortality in male patients. The optimal cut-off value for serum angiopoietin-2 to predict all-cause mortality in male patients was 99.1 pmol/L (AUC=0.69, 95%CI 0.62-0.76, p<0.0001). (B) Receiver operating characteristic (ROC) curves for all-cause mortality in female patients. The optimal cut-off value for serum angiopoietin-2 to predict all-cause mortality in female patients was 85.9 pmol/L (AUC=0.57, 95%CI 0.45-0.68, p=0.249).

3.3.4 Kaplan-Meier survival analysis

In Kaplan-Meier analysis, male patients with lower Ang-2 level (<111.0 pmol/L) showed a significantly higher survival rate than those with higher Ang-2 level (≥111.0 pmol/L) (log-rank test, p<0.0001), but this significant difference was not found in female patients (p=0.380).

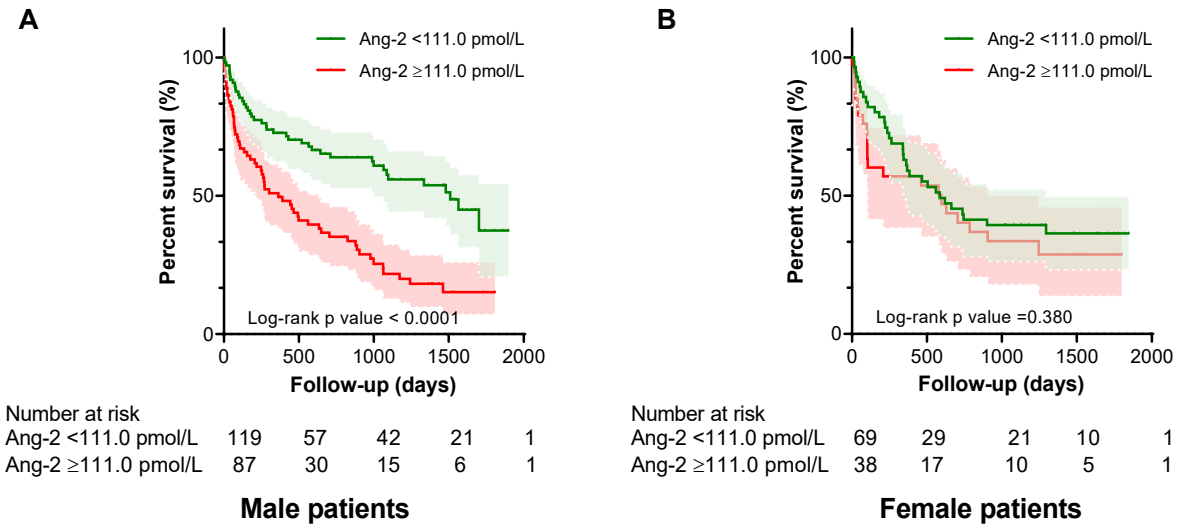

Figure 6. (A) Kaplan-Meier survival curves for male patients below and above optimal predictive value of angiopoietin-2 (111.0 pmol/L). (B) Kaplan-Meier survival curves for female patients below and above optimal predictive value of angiopoietin-2 (111.0 pmol/L). The shaded area represents the 95% confidence interval for the curve. This figure is modified from our published paper (44).

3.3.5 Cox regression analysis

In order to avoid potential bias in the estimated hazard ratios, binary Ang-2 was divided not only by the ROC-derived cut-off value of the entire participants (111.00 pmol/L), but also the ROC-derived cut-off value of males (99.1 pmol/L), females (85.9 pmol/L) and median values of Ang-2 (91.2 pmol/L). In Univariate Cox regression analyses, both increased Ang-2 and the binary upper half of the Ang-2 were positively and significantly associated with all-cause mortality in males, but this significance was not found in females. Furthermore, multivariable Cox regression analyses consistently showed a significant and independent association between Ang-2 level and all-cause mortality only in the males, not in the females (table 4).

Table 4. Multiple cox regression models analyzing serum angiopoietin-2 as a predictor of all-cause mortality in sex subgroups.

	Male (n=206)		Female (n=107)	
	HR (95% CI)	**P value**	**HR (95% CI)**	**P value**
Univariate Cox regression				
Continuous angiopoietin-2	1.002 (1.001-1.004)	0.009	1.003 (0.998-1.008)	0.221
Binary angiopoietin-2 [a]	2.475 (1.660-3.689)	<0.0001	1.273 (0.742-2.184)	0.381
Binary angiopoietin-2 [b]	2.449 (1.630-3.681)	<0.0001	1.049 (0.613-1.794)	0.863
Binary angiopoietin-2 [c]	2.227 (1.452-3.415)	0.0002	1.325 (0.772-2.274)	0.307
Binary angiopoietin-2 [d]	2.257 (1.491-3.415)	0.0001	1.041 (0.613-1.768)	0.880
Multivariable Cox regression [a]				
Model A	2.467 (1.624-3.747)	<0.0001	0.885 (0.503-1.555)	0.670
Model B	2.937 (1.660-5.196)	0.0002	1.990 (0.946-4.186)	0.070
Model C	3.294 (1.768-6.138)	0.0002	1.084 (0.476-2.467)	0.847
Multivariable Cox regression [b]				
Model A	2.368 (1.544-3.631)	<0.0001	0.764 (0.438-1.334)	0.344
Model B	2.700 (1.482-4.919)	0.001	1.447 (0.672-3.115)	0.345
Model C	2.614 (1.414-4.832)	0.002	0.569 (0.247-1.313)	0.186
Multivariable Cox regression [c]				
Model A	2.374 (1.524-3.697)	0.0001	0.968 (0.547-1.714)	0.912
Model B	2.627 (1.416-4.872)	0.002	1.822 (0.777-4.276)	0.168
Model C	3.103 (1.606-5.996)	0.001	0.492 (0.167-1.447)	0.197
Multivariable Cox regression [d]				
Model A	2.283 (1.475-3.533)	0.0002	0.801 (0.465-1.382)	0.425
Model B	2.393 (1.316-4.352)	0.004	1.887 (0.848-4.198)	0.120
Model C	2.553 (1.362-4.785)	0.003	0.618 (0.260-1.466)	0.275

[a] Binary Ang-2 was divided by ROC-derived cut-off value of the entire study population (111.0 pmol/L), [b] Binary Ang-2 was divided by ROC-derived cut-off value of male population (99.1 pmol/L). [c] Binary Ang-2 was divided by ROC-derived cut-off value of female population (85.9 pmol/L), [d] Binary Ang-2 was divided according to the median of the entire study population (91.2 pmol/L). Model A was adjusted for age, comorbidities (diabetes, hypertension, and coronary heart diseases), and smoking status. Model B was adjusted for dialysis vintage, serum creatinine, hemoglobin, C-reactive protein, serum albumin, ferritin, transferrin, iPTH, serum calcium, serum phosphorus, LDL, Kt/V. Model C was adjusted for all risk factors in model A and B, plus ultrafiltration volume. This table was modified from our published paper (44).

4. Discussion

4.1 Summary of results

The present study demonstrated that baseline circulating Ang-2 level is positively associated with all-cause mortality during a five-year follow-up in a cohort of patients with ESRD on HD treatment. Furthermore, this association between baseline Ang-2 levels and all-cause mortality is significant throughout various statistical method in males only. Our findings are based on several independent statistical methods,

1. The median baseline Ang-2 concentration was significantly lower in survivors than non-survivors ($p<0.0001$). A similar result was also seen in male patients ($p<0.0001$), but not in female patients ($p=0.249$).

2. In the survival analysis, the Kaplan-Meier curve suggested that male patients with the lower Ang-2 level (<111.0 pmol/l) had a significantly higher survival rate (log-rank test, $p<0.0001$), but not in female patients ($p=0.380$).

3. After multiple Cox regression analyses were performed, this sex-dependent impact on all-cause mortality was also found, i.e., elevated Ang-2 level was associated with all-cause mortality in males but not females on HD.

4.2 Angiopoietin-2 and all-cause mortality

The angiopoietin/Tie2 signaling axis plays a crucial role in regulating vascular integrity and quiescence (45, 46). The loss of vascular quiescence is a typical character of pathological conditions such as inflammation, atherosclerosis, hypoxia, high glucose, and various types of vasculopathy. Thus, this signaling is involved in numerous pathological situations which are associated with the destabilization of the endothelium (45).

We found a robust and significant association between baseline serum Ang-2 concentrations and all-cause mortality using several independent statistic approaches in this dialysis cohort. In multivariate Cox regression analysis, we adjusted for factors known to be associated with Ang-2 and mortality in ESRD on HD, i.e., age, comorbidities (diabetes, hypertension, and coronary heart diseases), smoking status, dialysis vintage,

serum creatinine, hemoglobin, C-reactive protein, serum albumin, ferritin, transferrin, iPTH, serum calcium, serum phosphorus, LDL, Kt/V and ultrafiltration volume. We observed a consistent statistical significance between Ang-2 concentrations and survival.

The association between blood Ang-2 and all-cause mortality has been studied in several different cohorts before. Lorbeer R et al. (47) reported that increased circulating Ang-2 level was associated with a higher risk for all-cause and cardiovascular mortality in a community-based study. In a retrospective case-control study, Ang-2 levels were associated with retinopathy and predicted mortality in Malawian children with cerebral malaria (48). Moreover, Allegretti AS et al. (49) reported that circulating Ang-2 levels were robustly associated with mortality and other clinically relevant outcomes in a cohort of decompensated cirrhotic patients with acute kidney injury. Additionally, Fisher J et al. (50) reported that elevated Ang-2 levels were associated with fluid overload, organ dysfunction, and increased mortality in human septic shock. In a population with chronic kidney diseases, David S et al. (30) demonstrated that elevated Ang-2 levels are able to predict long-term mortality, which are independent of conduit arterial stiffness or vascular calcification, over a four-year follow-up period in patients with stages 4-5 CKD. Later on, Tsai YC et al. (51) reported that in a cohort of 621 pre-dialysis stage 3-5 CKD patients, circulating Ang-2 level was an independent predictor of major adverse cardiovascular events and all-cause mortality. Considering previous studies, one might suggest that slight elevation in Ang-2 concentrations probably mirrors the vascular remodeling process related to a higher risk of mortality.

Ang-2 excess has been found in end-organ injury and hemodynamic alterations in independent studies (49, 52, 53). Ang-2 has a molecular weight of about 55 kDa, so it is very improbable that it will be cleared by glomerular filtration or dialysis clearance. (36, 37). Furthermore, these proteins exist as either dimers or tetramers and therefore are even larger in their native state (54). In HD patients, circulating Ang-2 concentration was elevated compared with healthy controls and pre-dialysis CKD populations (36, 37). However, the association between circulating Ang-2 levels and all-cause mortality has not been investigated. We performed several independent statistic approaches as previously described. In order to clarify this independent association, we performed multivariate Cox

regression analysis in multiple models. We adjusted for the risk factors known for ESRD patients' mortality on HD, and the factors associated with Ang-2, such as inflammatory situation (C-reactive protein), fluid overload (ultrafiltration volume) and cardiovascular diseases. We found a significant and consistent prognostic value of baseline serum Ang-2 levels in ESRD patients on HD.

4.3 Sex-specific findings

One important observation in our study is that elevated Ang-2 levels differ significantly in their ability to predict all-cause mortality in males and females. To the best of our knowledge, our study is the first one to present a sex-specific association between serum Ang-2 levels and all-cause mortality in the HD population.

Sexual dimorphism in circulating Ang-2 levels has been reported in a case-control study of obese versus non-obese individuals (55), as well as in the general population (56). This sex-related difference has also been noticed in other endothelial cell-derived growth factors, such as vascular endothelial growth factor (VEGF) and hepatocyte growth factor (57). However, these results were not identical; some reported increased level in males (58), while others observed elevated levels in females (57, 59).

We found a significant association between baseline Ang-2 levels and all-cause mortality. So next, we wondered and hence analyzed if there is a significant difference between males and females concerning Ang-2 level and if there is an association between baseline Ang-2 levels and all-cause mortality, which is sex-dependent based on the background described above. Interestingly, we found male patients have a non-significant higher level of baseline Ang-2 concentrations than females [94.30 (64.48, 143.78) pmol/L vs. 86.30 (59.30, 127.70) pmol/L, p=0.352], but a significant sex-related difference of the prognostic value of Ang-2 levels for all-cause mortality.

The mechanisms underlying sex-specific differences in circulating growth factors are still unclear so far. One explanation may be sex differences in endothelial function. Estrogen promotes the proliferation and survival of vascular ECs not only in reproductive tissues, but also in non-reproductive organs (60). It has been established that the vascular

system and reproductive tissue are both important targets for the direct effects of estrogen, and estrogen receptors have been identified in ECs (61). Previous experimental and clinical evidence suggests that estrogen may play a key role in the prevention or reversal of endothelial dysfunction by maintaining and increasing endothelial nitric oxide production and inhibiting endothelium-derived contractile factor (61). It is known that CVD is closely associated with endothelial dysfunction and has a greater incidence in males than females (62). Prior studies revealed that the cardiovascular benefits of estrogen could be attributed to the positive impacts on traditional CVD risk factors, such as lipoprotein profile, as well as the direct protective effects on vascular ECs (61, 62). However, androgens are often suspected to have detrimental effects on CVD (61). Furthermore, Tsuzuki T et al. (63) showed that female sex hormones regulate Ang-1, Ang-2, VEGF mRNA, and protein production in human endometrial stromal cells. Therefore, a possible hypothesis for the current sex-specific findings is that female sex hormones promote the survival of vascular ECs and regulate the angiopoietin/Tie2 signaling axis; this may result better regulation of circulating Ang-2 in females. However, males fall short of this additional regulation and therefore are probably more sensitive than females to Ang-2, which is involved in the pathogenesis of vascular inflammation, endothelial dysfunction and atherosclerosis and thus shows a significant association with all-cause mortality [44].

4.4 Study limitations

We acknowledge several limitations of the present study. First, this observational study makes it challenging to conclude causality and further independent validation is needed. Second, according to the ethics committee's approval, blood samples were only collected at the beginning of the study, so Ang-2 concentrations were only measured once at the beginning of the study. Therefore, the association of all-cause mortality and Ang-2 concentrations over time could not be analyzed. In addition, there was no pre-planned blinded study endpoint committee and only a few deaths where autopsies were performed at pathology institutes to clarify the cause of death. Thus, we do not have reliable data on

the cause of death to assess the association of Ang-2 concentrations with cardiovascular mortality.

4.5 Clinical significance

Our study suggests that circulating Ang-2 might be a worthwhile predictor of all-cause mortality in male ESRD patients on HD treatment. Furthermore, Ang-2 is probably not just a biomarker of mortality, but a player in the pathogenesis of vascular dysfunction in male ESRD patients [44]. Therefore, Ang-2 might be a therapeutic target to improve all-cause mortality in ESRD patients.

So far, a series of studies have suggested that Ang-2 antagonist has promising vasculoprotective effects and thus may offer a new therapeutic alternative. Majority data are from oncology (64). In preclinical data, Ang-2 antagonists successfully reduced tumor burden and angiogenesis and improved vascular stabilization (64). In the subsequent early-phase clinical trials, the safety and potential efficacy of several agents targeting the Ang-2-Tie2 pathway were proven, including Ang-2 inhibitor (MEDI3617, a human monoclonal antibody targeting Ang-2), Ang-1/2 inhibitor (Trebananib, a peptide fusion protein targeting Ang-1 and Ang-2), and Ang-2, VEGF-A inhibitor (Vanucizumab, a bispecific monoclonal antibody targeting Ang-2 and VEGF-A) (65).

In addition, Ang-2 blocking antibodies have likewise emerged as an attractive therapeutic target in many other diseases associated with Ang-2 dysregulation. Lee SJ et al. (66) reported that inhibition of Ang-2 significantly improved cardiac hypoxia and inflammation after ischemia injury in animal models. Moreover, therapeutic effects of Ang-2 blockade have also been found in atherosclerosis (67), non-alcoholic steatohepatitis (68), retinal vascular diseases (69) and organ transplantation (70). Given the significant and very consistent association between Ang-2 levels and all-cause mortality in male ESRD patients, our study may inspire the further development of Ang-2 antagonists in this population.

5 Conclusions

Circulating levels of Ang-2 are independently associated with all-cause mortality in male patients with ESRD on HD. Ang-2 may not only be a biomarker of all-cause mortality, but likely a potent vascular hormone that contributes to the pathophysiology of vascular injury in males with ESRD on HD. Present study after independent validation may inspire the development of Ang-2 antagonists to improve all-cause mortality in male ESRD patients.

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Statutory Declaration

"I, Chang Chu, by personally signing this document in lieu of an oath, hereby affirm that I prepared the submitted dissertation on the topic "Circulating Angiopoietin-2 level is independently associated with all-cause mortality in male end-stage kidney disease patients on hemodialysis", "Angiopoietin-2 ist ein unabhängiger Risikofaktor der Gesamtmortalität bei männlichen Hämodialysepatienten", independently and without the support of third parties, and that I used no other sources and aids than those stated.

All parts which are based on the publications or presentations of other authors, either in letter or in spirit, are specified as such in accordance with the citing guidelines. The sections on methodology (in particular regarding practical work, laboratory regulations, statistical processing) and results (in particular regarding figures, charts and tables) are exclusively my responsibility.

Furthermore, I declare that I have correctly marked all of the data, the analyses, and the conclusions generated from data obtained in collaboration with other persons, and that I have correctly marked my own contribution and the contributions of other persons (cf. declaration of contribution). I have correctly marked all texts or parts of texts that were generated in collaboration with other persons.

My contributions to any publications to this dissertation correspond to those stated in the below joint declaration made together with the supervisor. All publications created within the scope of the dissertation comply with the guidelines of the ICMJE (International Committee of Medical Journal Editors; http://www.icmje.org) on authorship. In addition, I declare that I shall comply with the regulations of Charité – Universitätsmedizin Berlin on ensuring good scientific practice.

I declare that I have not yet submitted this dissertation in identical or similar form to another Faculty.

The significance of this statutory declaration and the consequences of a false statutory declaration under criminal law (Sections 156, 161 of the German Criminal Code) are known to me."

Date Signature

Declaration of individual contribution to the publication

Chu C, Chen X, Hasan AA, Szakallova A, Krämer BK, Tepel M, Hocher B. Angiopoietin-2 predicts all-cause mortality in male but not female end-stage kidney disease patients on hemodialysis [published online ahead of print, 2021 Nov 18]. *Nephrol Dial Transplant*. 2021;gfab332. doi:10.1093/ndt/gfab332 (IF=5.992).

Contributions in detail:

I participated in the conception of the research idea and conducted all the data analysis of the study. Tables 1 and 2 were created based on my statistical evaluation. Table 1 showed baseline demographics and medical data of hemodialysis patients by sex and presented statistical differences between groups. Table 2 showed the results of cox regression analyses in multiple models of the entire cohort and also in the subgroups of males and females. In addition, I designed figure 1, plots of serum Ang-2 concentrations to show the difference in baseline Ang-2 levels in survivors and nonsurvivors, as well as in the subgroups of male survivors and nonsurvivors and female survivors and nonsurvivors and did statistical comparisons between groups. I created figure 2, plots of serum Ang-2 concentrations according to the underlying renal diseases. Moreover, I created Kaplan-Meier survival curves for all-cause mortality in the entire cohort, male patients and female patients based on the calculated optimal cut-off value of Ang-2 concentration (Figure 3). Furthermore, I conducted all supplementary data, including supplementary table 1, table 2, and supplementary figure 1 in publication. Finally, I wrote the original draft, and I did all additional analyses according to reviewers' comments, revised and submitted the final manuscript.

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Signature, date and stamp of first supervising university professor / lecturer

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Signature of doctoral candidate

Excerpt from Journal Summary List

Journal Data Filtered By: **Selected JCR Year: 2020** Selected Editions: SCIE,SSCI
Selected Categories: **"UROLOGY and NEPHROLOGY"** Selected Category Scheme: WoS
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Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
1	Nature Reviews Nephrology	10,033	28.314	0.019840
2	EUROPEAN UROLOGY	42,109	20.096	0.062260
3	Nature Reviews Urology	5,267	14.432	0.008470
4	KIDNEY INTERNATIONAL	52,616	10.612	0.039020
5	Kidney International Supplements	3,207	10.545	0.001970
6	JOURNAL OF THE AMERICAN SOCIETY OF NEPHROLOGY	45,533	10.121	0.047120
7	AMERICAN JOURNAL OF KIDNEY DISEASES	27,640	8.860	0.027100
8	Clinical Journal of the American Society of Nephrology	21,638	8.237	0.029300
9	European Urology Oncology	1,413	7.479	0.004350
10	JOURNAL OF UROLOGY	53,977	7.450	0.034650
11	EUROPEAN UROLOGY SUPPLEMENTS	1,023	7.122	0.001720
12	European Urology Focus	3,058	5.996	0.008120
13	NEPHROLOGY DIALYSIS TRANSPLANTATION	29,250	5.992	0.023440
14	Aging Male	1,521	5.892	0.001170
15	BJU INTERNATIONAL	23,873	5.588	0.019310
16	PROSTATE CANCER AND PROSTATIC DISEASES	3,161	5.554	0.005150
17	World Journal of Mens Health	948	5.400	0.001090
18	SEMINARS IN NEPHROLOGY	3,690	5.299	0.004100
19	Sexual Medicine Reviews	1,276	4.836	0.002770

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Angiopoietin-2 predicts all-cause mortality in male but not female end-stage kidney disease patients on hemodialysis

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ABSTRACT

Background. Angiopoietin-2 (Ang-2) plays a pivotal role in pathological vascular remodeling and angiogenesis. Both vascular mechanisms are active in patients with end-stage renal disease (ESRD) and may contribute to the high mortality in these patients. The aim of this multicenter prospective cohort study was to investigate baseline serum Ang-2 concentrations in ESRD patients on hemodialysis (HD) for their ability to predict all-cause mortality.

Methods. We conducted a prospective cohort study in 340 stable HD patients from different chronic dialysis centers in Berlin, Germany. The primary endpoint was all-cause mortality during a 5-year follow-up period. Blood samples and clinical data were collected at baseline. Serum Ang-2 was measured with a validated enzyme-linked immunosorbent assay (Biomedica, Vienna, Austria).

Results. A total of 313 HD patients (206 men and 107 women) were finally included in the study. Receiver operating characteristic (ROC) analysis of Ang-2 concentrations yielded an area under the curve (AUC) of 0.65 ($P < 0.0001$) for predicting all-cause mortality in the entire study population and was used to determine the optimal cut-off (111.0 pmol/L) for all-cause mortality. Kaplan–Meier survival analysis indicated that male but not female end-stage kidney disease patients on HD with higher Ang-2 concentrations had a significantly lower survival (log-rank test, $P < 0.0001$ and $P = 0.380$ for male and female patients, respectively). Multivariable Cox regression analyses adjusted for age, comorbidity, smoking, dialysis vintage, serum creatinine, hemoglobin, C-reactive protein, serum albumin, intact parathyroid hormone (iPTH), low-density lipoprotein (LDL) and Kt/V likewise indicated that elevated Ang-2 concentrations are associated with all-cause mortality in male [hazard ratio [HR] 3.294 [95% confidence interval (CI) 1.768–6.138]; $P = 0.0002$] but not in female end-stage kidney disease patients on HD [HR 1.084 (95% CI 0.476–2.467); $P = 0.847$].

Conclusion. Ang-2 at baseline is independently associated with all-cause mortality in male ESRD patients on HD.

Keywords: angiopoietin-2, all-cause mortality, hemodialysis patients, sex-dependent impact

INTRODUCTION

Angiogenesis is the process of forming new vessels on top of preexisting ones. It is involved not only in physiological conditions, but also in many pathological conditions with endothelial dysfunction/microinflammation features such as tumor metastasis and atherosclerotic plaque formation [1]. Angiopoietins (Angs) are vascular growth factors of ~70 kDa that are involved in angiogenesis and vasculogenesis and function through the Tie tyrosine kinase receptors. Ang-1 and Ang-2 regulate endothelial cell survival, angiogenesis and maturation through opposing functions. Ang-1 binds to the Tie2 receptor and induces Tie2 phosphorylation to provide an anti-inflammatory signal to the endothelium, thereby promoting endothelial cell survival and stabilizing endothelial and vascular structure, whereas Ang-2 competes with Ang-1 to bind to the Tie2 receptor and therefore destabilizes the vessel and degrades the basal lamina [2–4].

KEY LEARNING POINTS

What is already known about this subject?

- Angiopoietins (Angs) are vascular growth factors involved in angiogenesis and vasculogenesis.
- Angiopoietin-1 (Ang-1) binds to Tie tyrosine kinase 2 (Tie2) receptors and induces Tie2 phosphorylation–mediated anti-inflammatory signals to the endothelium, thereby promoting endothelial cell survival and stabilizing endothelial and vascular structure, whereas angiopoietin-2 (Ang-2) competes with Ang-1 to bind to the Tie2 receptor, thereby harming angiogenesis and vasculogenesis.
- In the general population, elevated Ang-2 concentrations are associated with a higher risk of all-cause and cardiovascular mortality.

What this study adds?

- Kaplan–Meier survival analysis indicated that male but not female end-stage kidney disease patients on hemodialysis (HD) with higher Ang-2 concentrations had a significantly lower survival (log-rank test, $P < 0.0001$ and $P = 0.380$ for males and females, respectively).
- Multivariable Cox regression analyses adjusted for age, comorbidity, smoking, dialysis vintage, creatinine, hemoglobin, C-reactive protein, albumin, intact parathyroid hormone, low-density lipoprotein and Kt/V likewise indicated that elevated Ang-2 concentrations are associated with all-cause mortality in male [hazard ratio [HR] 3.294 [95% confidence interval (CI) 1.768–6.138]; $P = 0.0002$] but not in female end-stage renal disease (ESRD) patients on HD ($P = 0.847$).

What impact this may have on practice or policy?

- The very strong sex dependency of the association of Ang-2 with all-cause mortality may stimulate further clinical and basic science studies.
- Ang-2 is a small bioactive molecule and hence not just an all-cause mortally biomarker, but rather likely a vascular powerful hormone contributing to the pathophysiology of vascular damage in male ESRD patients.
- Our study after independent confirmation might stimulate the development of Ang-2 antagonists to reduce all-cause mortality in ESRD patients.

Accumulating evidence has demonstrated that angiopoietins participate in cardiovascular burden. Elevated Ang-2 levels have been associated with traditional risk factors for cardiovascular diseases (CVDs), such as blood pressure, smoking, lipid levels and the metabolic syndrome [5, 6]. In the general population, elevated Ang-2 concentrations are associated with a higher risk of all-cause and cardiovascular mortality [7]. The kidney is a highly vascularized organ, characterized by a remarkable diversity of endothelial cells (ECs). The renal endothelium is both a target and a driver of kidney and systemic cardiovascular complications [8]. The Ang–Tie2 system has been shown to play a major role in injury induced by chronic kidney disease (CKD) and dialysis [9–11]. In CKS Stages 3–5 patients, high Ang-2 levels have been positively associated with systemic markers/mediators of inflammation [9, 10, 12, 13], abnormal cardiac structure [14] and major cardiac adverse events [15]. In patients with end-stage renal disease (ESRD), circulating Ang-2 levels have been found to be increased in patients treated with dialysis, although the mechanism is unknown. Furthermore, David *et al.* [9] suggested that Ang-2 might be a mediator and not just simply a biomarker of CVD events and thus accounts for accelerated atherosclerosis. They suggested that dialysis treatment is associated with severe disequilibrium of the Angs that probably confers permanent devastating activation of the endothelial layer.

Thus the aim of this study was to evaluate whether Ang-2 is associated with all-cause mortality in patients with ESRD on hemodialysis (HD).

MATERIALS AND METHODS

Study population

We conducted a prospective cohort study in 340 stable HD patients from different chronic dialysis centers in Berlin, Germany. The patients were followed up for 5 years. The study was approved by the local ethics committee and informed consent was obtained from all participants. Patients with any malignancy or active infections, pregnant or unwilling to take part were excluded from the study. All patients were routinely dialyzed at least three times a week for 4–5 h each time, using standard bicarbonate dialysis with biocompatible membranes. Dialysate flow rates were 500 mL/min and blood flow rates were 250–300 mL/min. All patients had a functioning permanent access. All-cause mortality was documented during the 5-year follow-up period. Patients who received a transplant during the follow-up period were censored at the time of transplantation.

Clinical data and serum parameters

The following patient characteristics were obtained: age, sex, weight, height, underlying renal disease, dialysis vintage, systolic and diastolic blood pressure (BP), presence of diabetes, hypertension, smoking or coronary heart disease and medications (use of angiotensin-converting enzyme inhibitors, beta-blockers, calcium channel blockers or erythropoietin). Blood samples were collected before one HD session at study entry. Serum albumin, cholesterol, triglycerides, urea,

creatinine, calcium, potassium and phosphate were assessed in the clinical laboratory using standardized methods. Ang-2 concentrations were analyzed using a sandwich enzyme immunoassay [human angiopoietin-2 enzyme-linked immunosorbent assay (ELISA), BI-ANG2, Biomedica, Vienna, Austria] according to the instructions of the manufacturer (https://www.bmgrp.com/wp-content/uploads/2019/11/BI-ANG2-Angiopoietin-2-ELISA-Validation-Data-191128.pdf). The limit of detection of the kit was 3.7 pmol/L, and the average intra- and interassay coefficients of variation were ≤8 and ≤6, respectively.

Statistical analysis

Descriptive variables are shown as median [interquartile range (IQR)] or number (percentage). Comparisons were assessed by Mann–Whitney U test or Kruskal–Wallis test, as appropriate. The cut-off value for all-cause mortality of baseline Ang-2 concentrations was obtained with receiver operating characteristic (ROC) curve analysis, with the value maximized by the Youden index. Cumulative survival curves were generated using the Kaplan–Meier method by optimal prediction value and differences were evaluated with a log-rank test. Multivariable-adjusted survival analysis was performed using a proportional hazards regression model. Model A was adjusted for age, comorbidities (diabetes, hypertension and CVD) and smoking; model B was adjusted for serum creatinine, hemoglobin, C-reactive protein (CRP), serum albumin, ferritin, transferrin, intact parathyroid hormone (iPTH), serum calcium, serum phosphorus, low-density lipoprotein (LDL) cholesterol and Kt/V (a number used to quantify HD and peritoneal dialysis treatment adequacy/quality, where K is the dialyzer clearance of urea, t is the dialysis time and V is the volume of distribution of urea, approximately equal to the patient's total body water); model C was adjusted for all of the above risk factors (model A + model B) plus ultrafiltration volume. Hazard ratios (HRs) and their 95% confidence intervals (CIs) were calculated. Furthermore, a series of multivariable Cox regression analyses was performed in these three models with three ROC-derived cut-off values: ROC-based cut-off for the entire study population (111.00 pmol/L), ROC-based cut-off for male participants (99.05 pmol/L) and ROC-based cut-off for female participants (85.85 pmol/L), as well as the median of male and female study participants (91.20 pmol/L). Statistical significance was defined as $P < 0.05$. All analyses were performed using SPSS version 25.0 (IBM, Armonk, NY, USA) and Prism 8 (GraphPad Software, San Diego, CA, USA).

RESULTS

Initially, 340 HD patients were included in the study, but 27 patients were excluded due to limited sample volume for Ang-2 measurements. Finally, 313 patients were included in statistical analysis: 206 male patients and 107 female patients. The median age was 66 years (IQR 56–75), the median time since the initiation of dialysis (dialysis vintage) was 243 days (IQR 31–1172) and the median dialysis dose (Kt/V) was 1.2 (IQR 1.1–1.3). During the 5-year follow-up, 157 patients (50.2%) died and 41 patients (13.1%) underwent kidney transplantation. The median baseline serum Ang-2 concentration was 91.2 pmol/L (IQR 63.4–140.7). Patients with an Ang-2 concentration above the median value at baseline were on average older; had longer dialysis vintage; had higher levels of ferritin, CRP, serum creatinine and potassium and had lower levels of transferrin and LDL than the patients with Ang-2 concentration below the median (Supplementary data, Table S1). The Ang-2 concentration was significantly higher in patients who died during follow-up than in those who did not die [112.5 pmol/L (95% CI 72.0–153.8) versus 78.6 (53.3–117.2); $P < 0.0001$]. The median serum Ang-2 was also significantly higher in the male nonsurvivors than the male survivors [119.8 (IQR 77.0–161.6) versus 78.4 (51.5–118.5); $P < 0.0001$], but not in female survivors and female nonsurvivors [91.2 (IQR 67.2–142.9) versus 80.2 (59.0–117.7); $P = 0.249$] (Figure 1).

Table 1 summarizes the baseline clinical and biochemical variables for patients by sex. The cause of CKD was hypertensive nephropathy in 107 cases (34.2%), diabetic nephropathy in 97 cases (31.0%), glomerulonephritis in 25 cases (8.5%), polycystic kidney disease in 9 cases (2.9%) and other or unknown in 75 cases (24.0%). Males comprised 65.8% of the cohort (206 males, 107 females). Females were less likely to be diabetics and smokers than males and had higher high-density lipoprotein cholesterol, total cholesterol and dialysis doses. Males had higher CRP and serum creatinine than females. However, there were neither sex differences nor differences among the different groups according to the underlying renal diseases, such as hypertensive renal diseases or diabetic nephropathy, in baseline Ang-2 concentrations (Figure 2).

The optimal cut-off value for baseline serum Ang-2 to predict all-cause mortality was 111.0 pmol/L (AUC 0.65, $P < 0.001$; sensitivity 0.522, specificity 0.727, Youden index value 0.250) based on the ROC analysis (Supplementary data, Figure S1).

Kaplan–Meier survival analysis indicated that patients in the higher Ang-2 concentration group (≥111.0 pmol/L) had a significantly lower survival rate (log-rank test, $P < 0.0001$), with similar results for male patients (Figure 3).

Next we performed univariate and multivariable Cox regression analyses. Binary Ang-2 was divided according to the optimal cut-off value (ROC-based cut-off for the entire study population 111.00 pmol/L). Univariate Cox regression analyses showed that both increasing Ang-2 and the binary upper half of Ang-2 were positively associated with all-cause mortality in both overall and male patients. Multivariable Cox regression analyses were then performed in three models (as described in the Materials and Methods section) (Table 2). Furthermore, multivariable Cox regression analysis was also performed in three models with ROC-derived cut-off values in sex subgroups (ROC-based cut-off for male participants 99.05 pmol/L, ROC-based cut-off for female participants 85.85 pmol/L) as well as the median of male and female study participants (91.20 pmol/L).

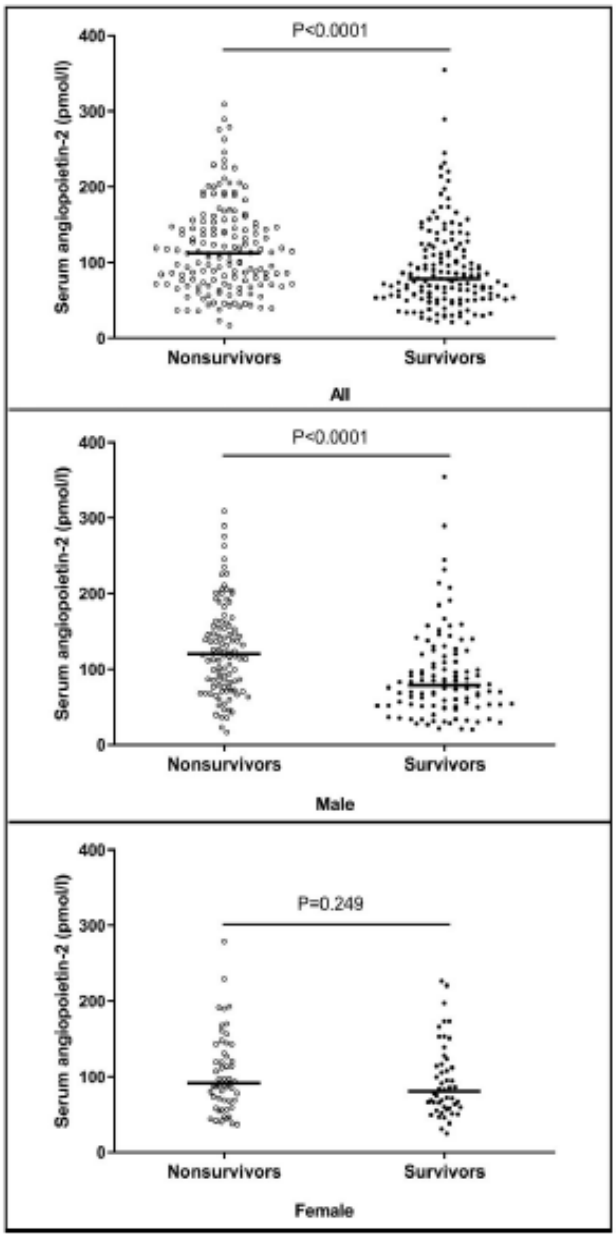

FIGURE 1: Plots of serum Ang-2 concentrations. Median serum Ang-2 was significantly higher in the nonsurvivors than the survivors using Mann–Whitney U test [112.50 (IQR 71.95–153.80) versus 78.55 (53.33–117.20); P < 0.0001]. Median serum Ang-2 was significantly higher in the male nonsurvivors than the male survivors using Mann–Whitney U test [119.80 (IQR 76.98–161.60) versus 78.40 (51.45–118.50); P < 0.0001]. No significant difference of median serum Ang-2 was found between female nonsurvivors and female survivors using Mann–Whitney U test [91.20 (IQR 67.20–142.90) versus 80.20 (59.00–117.70); P = 0.249].

Table 1. Baseline clinical and biochemical characteristics of HD patients by sex

Characteristics	All ($N = 313$)	Male ($n = 206$)	Female ($n = 107$)	P-value
Age (years)	66.00 (56.00–75.00)	66.00 (56.00–74.00)	67.00 (57.00–76.00)	0.617
Primary kidney diseases, *n* (%)				0.722
Hypertensive nephropathy	107 (34.2)	76 (36.9)	31 (29.0)	
Diabetic nephropathy	97 (31.0)	57 (27.7)	40 (37.4)	
Glomerulonephritis	25 (8.0)	13 (6.3)	12 (11.2)	
Polycystic kidney disease	9 (2.9)	7 (3.4)	2 (1.9)	
Other or unknown cause	75 (24.0)	53 (25.7)	22 (20.6)	
Diabetes mellitus, *n* (%)	117 (37.4)	66 (32.0)	51 (47.7)	0.007
Hypertension, *n* (%)	246 (78.6)	160 (77.7)	86 (80.4)	0.580
CHD, *n* (%)	148 (47.3)	99 (48.1)	49 (45.8)	0.704
Smoker, *n* (%)	97 (31.0)	75 (36.4)	22 (20.6)	0.004
Body mass index (kg/m^2)	24.53 (22.01–27.55)	24.91 (22.25–27.35)	24.12 (21.60–28.51)	0.306
Dialysis vintage (days)	243 (31–1172)	271 (31–1347)	227 (31–919)	0.180
Medication, *n* (%)				
RAAS inhibitors	82 (26.2)	54 (26.2)	28 (26.2)	0.993
Beta-blockers	186 (59.4)	116 (56.3)	70 (65.4)	0.120
Calcium channel blockers	98 (31.3)	65 (31.6)	33 (30.8)	0.897
Erythropoietin	158 (50.5)	104 (50.5)	54 (50.5)	0.998
Hemoglobin (mg/dL)	10.20 (9.05–11.45)	10.10 (9.00–11.20)	10.20 (9.15–11.60)	0.593
Ferritin (ng/ml)	516.50 (243.50–1074.00)	513.00 (239.00–1150.50)	520.00 (243.00–914.00)	0.762
Transferrin (µg/ml)	138.00 (107.00–173.00)	138.00 (104.00–172.00)	142.00 (116.25–177.00)	0.286
Serum albumin (g/L)	3.30 (2.90–3.70)	3.30 (2.90–3.70)	3.20 (2.80–3.60)	0.565
LDL (mg/dL)	92.70 (72.35–120.08)	92.70 (70.40–114.23)	103.00 (75.78–128.60)	0.101
HDL (mg/dL)	39.80 (32.00–50.20)	36.45 (30.90–46.32)	43.80 (36.43–56.68)	<0.001
Total cholesterol (mg/dL)	150.60 (127.40–187.00)	146.70 (119.70–182.25)	166.00 (134.15–204.00)	0.020
Triglycerides (mmol/L)	159.30 (109.50–247.80)	159.30 (106.20–247.80)	162.80 (112.40–247.80)	0.708
CRP (mg/dL)	2.60 (1.00–5.00)	3.10 (1.15–5.95)	1.40 (0.67–3.33)	<0.001
Urea (mg/dL)	195.52 (147.31–268.57)	205.09 (145.94–279.53)	183.18 (150.67–242.39)	0.110
Serum creatinine (mg/dL)	6.66 (4.25–8.39)	6.90 (4.56–8.71)	5.74 (3.76–7.46)	0.004
Serum potassium (mmol/L)	4.70 (4.10–5.26)	4.80 (4.10–5.30)	4.60 (4.00–5.20)	0.186
Serum calcium (mmol/L)	2.22 (2.09–2.40)	2.25 (2.08–2.40)	2.20 (2.10–2.44)	0.774
Serum phosphorus (mg/dL)	1.61 (1.20–2.10)	1.63 (1.21–2.10)	1.59 (1.17–2.01)	0.485
iPTH (ng/L)	50.18 (18.96–129.60)	47.71 (18.96–131.88)	52.79 (18.85–127.40)	0.769
Dialysis dose (Kt/V)	1.19 (1.07–1.33)	1.16 (1.06–1.31)	1.24 (1.11–1.44)	0.017
Ang-2 (pmol/L)	91.20 (63.35–140.65)	94.30 (64.48–143.78)	86.30 (59.30–127.70)	0.352

Values are presented as median (IQR) unless stated otherwise. Between groups (male versus female), comparisons were made using a nonparametric Kruskal–Wallis test for continuous variables and the χ^2 test for categorical variables. Body mass index was calculated as weight in kilograms divided by height in meters squared. CHD, coronary heart disease; RAAS, renin–angiotensin–aldosterone system.

Results were consistent in all models and revealed a significant predictive effect of Ang-2 on all-cause mortality in the whole cohort as well as in the male population, but not in the female population (Supplementary data, Table S2).

DISCUSSION

We assessed the association of circulating Ang-2 at baseline with all-cause mortality during a follow-up of 5 years in a multicenter HD cohort of patients with ESRD. Baseline serum Ang-2 concentrations showed a consistent positive association with all-cause mortality and this association was only seen in male HD patients.

Ang-2 is a secreted glycoprotein, synthesized mainly by endothelial cells but also by other cell types, that plays a critical role in vascular development. It mediates its effect via inhibition of Ang-1-mediated phosphorylation of Tie2. Ang-1 binds to Tie2 receptor and induces Tie2 phosphorylation to provide an anti-inflammatory signal to the endothelium, therefore Ang-2 release leads to inflammation and is associated with a range of pathological conditions [2–4]. In observational studies, circulating Ang-2 levels are elevated in a variety of diseases known for their common characteristics of endothelial dysfunction and/or vascular inflammation, such as diabetes mellitus [16], cardiovascular diseases [14, 17], systemic lupus erythematosus [18] and systemic inflammatory response syndrome (SIRS) [19–22]. Moreover, Ang-2 levels were positively associated with cardiovascular disease risk factors, including metabolic syndrome [5]. In CKD patients, Ang-2 levels are associated with systemic markers/mediators of inflammation. In addition, circulating Ang-2 increases with the progression of CKD, which is predictive of mortality and correlates with the severity of vascular disease in dialysis patients [9, 10, 13]. Previous studies have found that glomerular Ang-2 is upregulated in preclinical models of glomerulonephritis [23] and podocyte-specific expression of Ang-2 causes proteinuria [24], suggesting that the mechanism linking endothelial dysfunction, albuminuria and CVD is that of endothelial dysfunction, leading to increased vascular permeability and glomerular albumin leakage [25]. ESRD patients on HD have a higher incidence of CVD. In addition to conventional cardiovascular risk factors such as age, sex

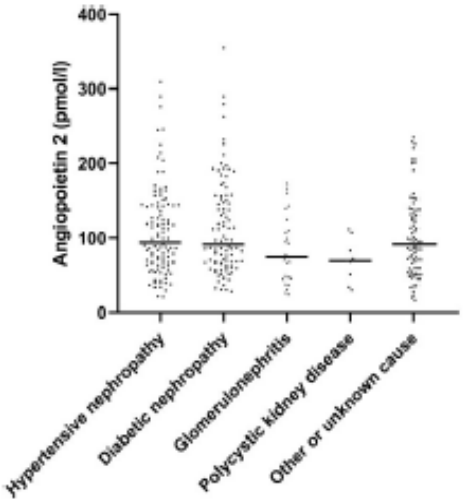

FIGURE 2: Plots of serum Ang-2 concentrations according to the underlying renal diseases. Lines indicate the median value of the Ang-2 concentration in each group.

and comorbidities, risk factors such as chronic inflammation and endothelial dysfunction highlight the impact of damage to the vascular endothelium in this complex pathophysiology. Endothelial dysfunction is associated with a higher incidence of CVD, which may be one of the main causes of the high morbidity and mortality in ESRD patients on HD. In the present study, we found a robust and consistent association between serum Ang-2 and all-cause mortality even after adjusting for multiple risk factors, i.e., age, comorbidities, smoking, dialysis vintage, serum creatinine, hemoglobin, CRP, serum albumin, ferritin, transferrin, iPTH, serum calcium, serum phosphorus, LDL cholesterol, *Kt/V* and ultrafiltration volume.

To our knowledge, the present study is the first to demonstrate a sex-dependent association between elevated Ang-2 concentrations and all-cause mortality. This finding is based on several independent statistical approaches. Kaplan–Meier survival analysis showed that male patients in the higher Ang-2 concentration groups (≥111.0 pmol/L) had a significantly lower survival rate (log-rank test, $P < 0.0001$), but not female patients. Multivariable Cox regression models revealed that Ang-2 has a sex-dependent impact on all-cause mortality in patients with ESRD on HD. Sexual dimorphism has been reported for serum Ang-2 levels in a relatively small case–control study of obese versus nonobese individuals [26] and in the general population [5]. These sex-related differences have also been observed in other endothelium-derived growth factors, such as vascular endothelial growth factor (VEGF) and hepatocyte growth factor [27]. However, some reported higher levels in males [28], whereas some observed increased levels in females [27, 29]. The mechanisms for the sex-related differences in circulating growth factors are not fully understood. Previous experimental and clinical evidence indicates that at least a part of cardiovascular benefits of 17β-estradiol can be attributed to the direct effect of the ovarian sex steroid hormone on vascular endothelial cells [30]. Furthermore, experimental studies suggest that sex hormones influence vascular growth factor expression [31]. Tsuzuki *et al.* [32] showed that sex hormones are involved in the regulation of Ang-1, Ang-2 and VEGF messenger RNA and protein production in human endometrial stromal cells. Teubner *et al.* [33] demonstrated a proangiogenic effect of testosterone, which may be due to stimulation of Ang-2 and transforming growth factor α expression [33]. A potential explanation for our sex-specific finding is that in ESRD patients on HD, males may be more sensitive than females to Ang-2, which is involved in the pathogenesis of vascular inflammation, endothelial dysfunction and atherosclerosis and therefore shows a significant association with all-cause mortality.

Our study, after independent confirmation, might stimulate the development of Ang-2 antagonists to reduce all-cause mortality in ESRD patients. Ang-2 is a small molecule acting as a paracrine endothelial hormone with a preside molecular target, thus being most likely not just a mortality biomarker, but rather a player in the pathogenesis of vascular damage in male ESRD patients. Given the very strong association with mortality in male ESRD patients, our study might stimulate the development of Ang-2 antagonists.

So far, several studies have shown that Ang-2 blockade alleviates pathological angiogenesis and hence might offer a novel therapeutic approach to treatment. Most data so far are coming from oncology. Preclinical studies indicate that blockade of Ang-2 with humanized monoclonal antibodies inhibited angiogenesis and tumor growth and induced vascular regression in multiple tumor models [34, 35]. In addition to compelling preclinical data, inhibition of Ang-2-Tie2 has been evaluated in numerous early clinical trials and demonstrated safety and potential efficacy for antitumor activity [36–39]. Furthermore, this antiangiogenic efficacy also produced encouraging results in preclinical studies in myocardial infarction [40] and nonalcoholic steatohepatitis [41] as well as in clinical trials in different retinal vascular disease models [42–45].

We acknowledge limitations of our study. First, this study is an observational epidemiologic study, which makes it difficult to draw conclusions on causality, and results should be validated in an independent cohort. The blood samples for this study were taken according to the approval of the ethical committee only at study entry, thus the Ang-2 concentrations were only measured once at study entry, hence it is impossible to investigate the association of mortality with the longitudinal profile of Ang-2 concentrations. In addition, we have no data on cardiovascular events or cardiovascular mortality and therefore cannot clarify whether this prognostic significance of Ang-2 is independent of underlying vascular disease or is associated with coronary atherosclerosis.

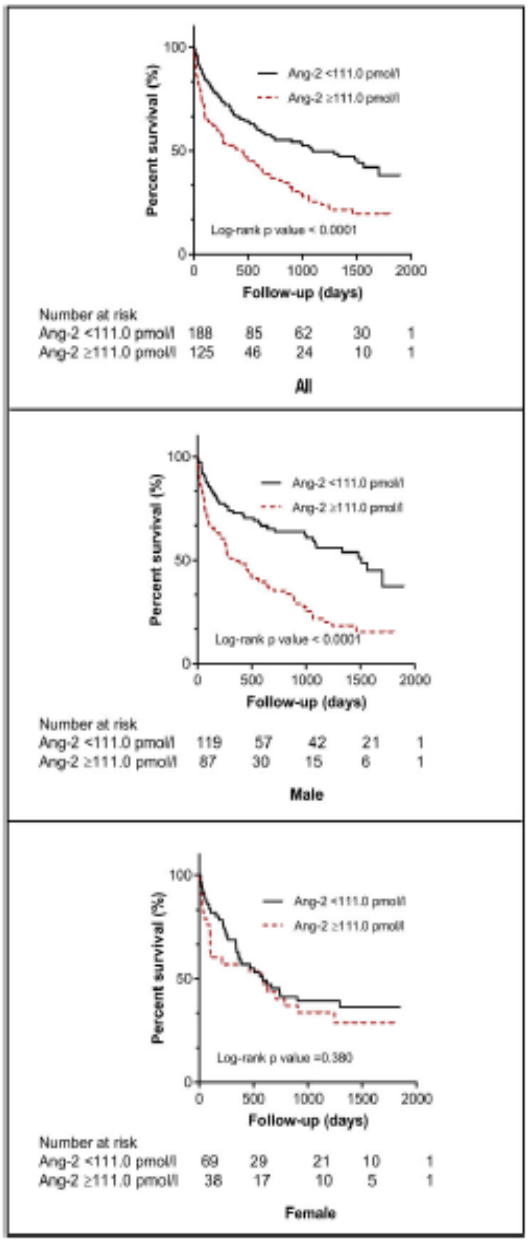

FIGURE 3: Kaplan–Meier survival curves for all-cause mortality. Patients were divided according to optimal cut-off values of Ang-2 concentrations.

Table 2. Cox regression analyses of serum Ang-2 levels predicting all-cause mortality

Analyses	All ($N = 313$) HR (95% CI)	P-value	Male ($n = 206$) HR (95% CI)	P-value	Female ($n = 107$) HR (95% CI)	P-value
Univariate Cox regression						
Continuous Ang-2	1.002 (1.001–1.004)	0.005	1.002 (1.001–1.004)	0.009	1.003 (0.998–1.008)	0.221
Binary Ang-2	1.934 (1.412–2.649)	<0.0001	2.475 (1.660–3.689)	<0.0001	1.273 (0.742–2.185)	0.381
Multivariable Cox regression						
Model A	1.756 (1.274–2.419)	0.001	2.467 (1.624–3.747)	<0.0001	0.885 (0.503–1.555)	0.670
Model B	2.613 (1.698–4.023)	<0.0001	2.937 (1.660–5.196)	0.0002	1.990 (0.946–4.186)	0.070
Model C	2.245 (1.443–3.493)	0.0003	3.294 (1.768–6.138)	0.0002	1.084 (0.476–2.467)	0.847

Binary Ang-2 was divided according to optimal cut-off values of Ang-2 concentrations (111.0 pmol/L). Multivariable Cox regression analyses were performed in three models. Model A was adjusted for age, comorbidity and smoking; model B was adjusted for dialysis vintage, serum creatinine, hemoglobin, CRP, serum albumin, ferritin, transferrin, iPTH, serum calcium, serum phosphorus, LDL and *Kt/V* and model C was adjusted for the above risk factors (model A + model B) plus ultrafiltration volume.

CONCLUSION

In conclusion, Ang-2 at baseline is independently associated with all-cause mortality in male ESRD patients on HD.

SUPPLEMENTARY DATA

Supplementary data are available at *ndt* online.

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AUTHORS' CONTRIBUTIONS

B.H. contributed to the research idea and study design. A.S. and M.T. were responsible for data acquisition. C.C., X.C. and A.A.H. were responsible for data analysis. C.C. and B.H. were responsible for article drafting. B.H., B.K.K. and M.T. were responsible for supervision or mentorship. All authors took part in the interpretation of the results and approved the final version of the manuscript.

CONFLICT OF INTEREST STATEMENT

The authors have no conflicts of interest to declare.

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Curriculum Vitae

My curriculum vitae does not appear in the electronic version of my paper for reasons of data protection.

Complete personal publications

1. **Chu C**, Delic D, Alber J, Feger M, Xiong Y, Luo T, Hasan AA, Zeng S, Gaballa MMS, Chen X, Yin L, Klein T, Elitok S, Krämer BK, Foller M, Hocher B. Head-to-head comparison of two SGLT-2 inhibitors on AKI outcomes in a rat ischemia-reperfusion model. Biomed Pharmacother. 2022;153:113357. (IF= 7.419)
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