Plasma Endothelin in Patients With End-Stage Renal Disease on Hemodialysis

PLASMA ENDOTHELIN IN PATIENTS WITH END-STAGE RENAL DISEASE ON HEMODIALYSIS

by

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I have reviewed this thesis. It represents work done by the author under my guidance/supervision.

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Background and context

Endothelin was identified in 1988 and has been widely investigated both in physiology and pathological mechanisms of a variety of diseases (1, 2). Endothelin-1 (ET-1) is the most biologically active and abundant member of the endothelin family, which consists of three peptides: ET-1, ET-2, and ET-3 (3). All three types of endothelin bind to two receptor isoforms, ETA and ETB, which share similar structures and mediate different effects (4). ET-1 has been shown to be involved in the pathogenesis of hypertension, atherosclerosis, and also to the development of chronic kidney diseases such as lupus nephritis, diabetic nephropathy, and polycystic kidney disease. (5) Elevated baseline ET-1 levels were associated with a higher incidence of chronic kidney disease (CKD) from the results of the Jackson Heart Study (6). Also, Plasma ET-1 levels are increased in CKD and end-stage renal disease compared with healthy individuals, and influence blood pressure (BP) regulation in ESRD. (7, 8) But the reasons for the higher ET-1 level and the significance of elevated ET-1 in individuals on hemodialysis are unclear.

ET-1's effects can be antagonized with selective and non-selective endothelin receptor antagonists. These drugs have been tested in a variety of contexts, including heart failure and coronary artery disease. They have also been tested in diabetic nephropathy. The effects of non-selective endothelin receptor antagonist showed exciting benefits of slowing kidney function decline in kidney disease animal models, but the human trial did not show promising results mainly due to the side effect such as fluid overload (9-11). ET_A receptor antagonist, on the other hand, did demonstrate efficacy in proteinuria reduction in diabetic nephropathy (12). The ESRD population is unique in that the main side effect of

ET_A receptor antagonism – which is volume overload, mediated by fluid retention by intact kidneys – would not be expected in ESRD patients treated with ET-1 receptor antagonists. To determine whether higher ET-1 levels associate with a higher risk of mortality and hospitalization ESRD, and what factors are associated with higher ET-1 levels, we measured plasma ET-1 levels in a cohort study involving patients on chronic maintenance hemodialysis. Finding a prognostic role for ET-1 may suggest ET-1 antagonism as a therapeutic approach in ESRD.

Paper 1: Plasma Endothelin-1 and the Risk of Death and Hospitalization in End Stage Renal Disease Patients on Hemodialysis

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Running Title: ET-1 in hemodialysis patients

Abstract

End-stage renal disease (ESRD) is a worldwide public health problem. The main treatment for ESRD is still hemodialysis (HD). Despite the substantial improvements in dialysis therapy, HD patients continue to experience significant mortality and morbidity. Endothelin-1 (ET-1) is a potent endothelium-derived vasoconstrictor peptide implicated in the pathogenesis of hypertension, congestive heart failure, and inflammation, all of which are key pathophysiologic features of end-stage renal disease (ESRD). In hemodialysis patients, ET-1 increases strikingly but the association of ET-1 with adverse outcomes in individuals with end stage renal disease on hemodialysis is unclear. To test the hypothesis that increased ET-1 levels are associated with increased adverse events in hemodialysis patients, we measured plasma ET-1 levels in a cohort of 794 individuals with prevalent ESRD treated with maintenance hemodialysis. The primary outcomes were time to death and hospitalization. The median plasma ET-1 level was 2.02 (interquartile range, 1.57 – 2.71) pg/mL. Compared to individuals in the lowest quartile of plasma ET-1, those in the highest quartile had a 2.52-fold higher risk of death (HR 2.52, 95% CI 1.68 – 3.79) and a 1.13-fold increased risk of hospitalization (HR 1.16, 95% CI 1.04 – 1.23) in multivariable models adjusting for demographic, clinical, and laboratory variables. Higher plasma ET-1 appears to be associated with adverse events in hemodialysis patients independent of previously described risk factors. Future trials are expected to test the potential role for ET-1 antagonists as a pharmacological intervention in ESRD.

Introduction

Hemodialysis (HD) is a life-sustaining therapy for patients with end-stage renal disease (ESRD). Despite advances in HD technologies and medical care, patients with ESRD on HD remain at markedly increased risk of death, most commonly due to cardiovascular disease (CVD). The reasons for accelerated mortality and high CVD risk in the ESRD population are not well explained. (13-16) Contributing factors include the high prevalence of hypertension; congestive heart failure, inflammation, and volume overload (17). Although many studies have identified some risk factors associated with mortality in hemodialysis patients, risk prediction and mortality reducing therapies in hemodialysis patients are inadequate (14, 15).

Endothelin-1 (ET-1) is a potent endothelium-derived vasoconstrictor peptide implicated in the pathogenesis of hypertension, congestive heart failure, and inflammation, all of which are key pathophysiologic features of end-stage renal disease (ESRD) (2). ET-1 acts in both autocrine and paracrine fashion by binding to two receptor isoforms, ETA and ETB, which mediate distinct effects on vascular tone and cell growth (18). ET-1 contributes to the pathogenesis of several cardiovascular disorders including hypertension and atherosclerosis, and also to the development of chronic kidney diseases (19). Plasma ET-1 levels are increased in CKD and end-stage renal disease compared with healthy individuals, and influence blood pressure (BP) regulation in ESRD. Endothelin receptor antagonists were developed to interfere with maladaptive endothelin receptor-mediated cell function and are currently in clinical use for the treatment of pulmonary arterial hypertension and under investigation in diabetic kidney disease (12, 20). A major side effect of ET-1

antagonists observed in clinical trials has been fluid overload, mediated by ET-1's effects on sodium reabsorption. Because volume regulation in HD patients is not mainly mediated by the kidneys but rather by the HD procedure, ET-1 antagonists would not be expected to significantly contribute to volume overload in HD patients. To explore the potential relevance of ET-1 and endothelin antagonism in ESRD, who has an extraordinarily high prevalence of hypertension and vascular disease, we measured plasma ET-1 levels in a cohort study involving prevalent hemodialysis patients. We hypothesized that ET-1 levels would associate with a higher risk of mortality and hospitalization. The primary outcomes were time to death and disease hospitalization.

Methods

Study design and population

The anonymized plasma samples and statistically de-identified clinical data obtained from a biorepository assembled by DaVita Clinical Research (Minneapolis, MN, USA). Anonymized plasma samples and statistically deidentified data were made available for academic research. The DaVita Clinical Research biorepository comprises blood samples and clinical data from 4,028 individuals with prevalent end-stage renal disease who received hemodialysis at a large dialysis organization (LDO) between May 2011 and October 2013. The biorepository sampling protocol was reviewed and approved by an Institutional Review Board (Quorum IRB, Seattle, WA, USA) and patients provided written informed consent prior to the initiation of sample collection. Patients with hemoglobin < 8.0 g/dL, who were < 18 years of age, who were pregnant, or who had any physical, mental, or medical condition which prohibited the ability to provide informed consent were

excluded from participation. The study met minimal risk criteria as set forth and defined at 21 CFR 56.111.

Biospecimen collection and storage

Under the biorepository study protocol, blood samples were collected from each subject at baseline and, thereafter, every three months for up to one year. Pre-dialysis blood samples were collected and processed according to a standardized protocol: specimens were shipped on refrigerated packs on the day of collection to a centralized laboratory, where they were aliquotted and stored at -80C. Specimens with cause for rejection (e.g., unspun tubes, insufficient volume, or thawed specimens) or that were received >48h from the time of collection were rejected. Anonymized plasma samples were shipped from the centralized laboratory to the researchers on dry ice at -80°C.

Measurement of Endothelin-1 (ET-1)

I measured plasma ET-1 levels in plasma samples in duplicate using a commercially available ELISA kit (Quantikine Human ET-1 Immunoassay PDET100; R&D Systems, Minneapolis, MN). Since ET-1 is unstable, I performed the procedure quickly as outlined below: 1) dissolve the blood samples at 37℃ for 5 min; 2) centrifuge at 3000r/min for 5 min, 4℃; 3) remove the supernatant to 96 wells microplate (master plate). 4) Prepare 1:2 Diluted plate: add 150 ul plasma + 150 ul Calibrator Diluent RD5-48 with one duplicated for each sample. 5) add the reagents into the well as protocol from the ELISA kits. Procedure 3 and 4 need to be done on ice. We assessed the inter-assay

coefficient of variation using 42 blind splits replicate plasma samples obtained from ESRD patients recruited at Brigham and Women's Hospital. The mean inter-assay CV from blind split replicates was 7.3%.

Outcome Ascertainment

The two primary outcomes were time to all-cause mortality and time to the first hospitalization, starting from the time of the first study blood draw. Vital status and hospitalization data were based on records obtained from DCR. For mortality analyses, patients were censored at the time of kidney transplantation (n=37), transfer to a non-affiliated dialysis unit (n=31), recovery from dialysis (n=3), and at the end of follow-up (December 31, 2014). For hospitalization analyses, patients were additionally censored at death.

Assessment of Other Covariates

Clinical and hemodialysis treatment data for each biorepository subject were collected by the LDO during routine care and were maintained in the LDO electronic health record. Clinical and hemodialysis treatment data were provided to the researchers by DaVita Clinical Research in statistically deidentified form. We collected data on demographics, clinical conditions, hemodialysis prescription data, comorbidities, and laboratory data. Laboratory tests were measured on blood samples collected pre-dialysis, except for post-dialysis BUN. Ultrafiltration volume was assessed by weight difference before vs. after the hemodialysis. All baseline covariate data were chosen according to the collection time point closest to the first study sample blood draw date.

Analytic approach

All analyses were performed in R version 3.4.2. We expressed continuous variables as means (standard deviations) or medians (IQR, 25th and 75th percentiles). Baseline characteristics were compared across quartiles of ET-1 using Kruskal Wallis for continuous variables and chi-squared tests for categorical variables. We used Cox proportional hazard models to examine the unadjusted and multivariable-adjusted risks of outcomes and modeled ET-1 in quartiles (with quartile one as the reference group) and as a continuous variable (per 1 unit SD of natural-log transformed plasma ET-1). Schoenfeld residuals were used to verify the proportional hazards assumption. Non-significant relationship between residuals and time were found for each of the covariates, and the global test was also not statistically significant. The cumulative incidence of death during follow-up was estimated by the Kaplan–Meier method, and the log-rank test was used for univariate analyses. Our multivariable adjustment strategy was hierarchical and based on biological and clinical plausibility as well as observed correlations of ET-1 with factors that could confound the association between ET-1 and outcomes. We fitted a series of multivariable models: Model 1: unadjusted; Model 2: adjusted for age, race, sex, BMI, SBP, diabetes as cause of ESRD, hemodialysis access, vintage, history of CVD; Model 3: Model 2 + albumin, ferritin, single pool eKt/V; Model 4 (exploratory): Model 3 + iron, TIBC, LDH, hemoglobin, WBC, PLT, RDW, PTH, phosphate, calcium. Effect modification of the association between continuous ET-1 and mortality by prespecified covariates (age, sex, race, BMI, dialysis vintage, with/without diabetes and with/without cardiovascular disease) was tested by including multiplicative interactions terms in the multivariable model. We tested whether ET-1 improved risk prediction by using the likelihood ratio test (LRT) to compare models with

vs. without ET-1. We examined time-dependent receiver operating characteristics (ROC) curves (cumulative case/dynamic control ROC by next neighbor estimation method) in multivariable model 4 with and without log-ET. (21) Complete data sets on variables for outcome analyses were available in 97% of the cohort. W We found no appreciable difference in results with multiple imputations by R package "mice" and therefore missing data were not imputed in the primary analysis. All statistical tests were two-sided, and P values < 0.05 were considered statistically significant for time to event analyses.

Results

Study population characteristics

A total of 794 participants were included in this study. At baseline, mean age was 60.0 $\pm$ 13.8 years, median dialysis vintage was 37.2 months (interquartile range (25-75th percentile, IQR), 18.1 to 72.6 months), and 58% were men. The most common reasons for ESRD were diabetes mellitus (45.0%), hypertension (31.9%), and other glomerular diseases (12.0%). The median plasma ET-1 level was 2.02 (IQR,1.57 – 2.71) pg/mL. Participants in higher quartiles of plasma ET-1 were younger, had higher systolic and diastolic blood pressures, longer dialysis vintage, and greater ultrafiltration volume (Table 1).

Table 1. Baseline characteristics of participants according to quartiles of plasma endothelin-1

	Quartile 1 n=199	Quartile 2 n=199	Quartile 3 n=197	Quartile 4 n=199	P value
Plasma endothelin-1, pg/mL	1.30 (1.10, 1.42)	1.78 (1.67, 1.88)	2.32 (2.14, 2.51)	3.33 (2.97, 4.06)	-
Age, years	62.80 (15.0)	60.1 (14.0)	59.8 (13.7)	57.7 (12.0)	0.003
Male, n (%)	121 (60.8)	110 (55.3)	118 (59.9)	112 (56.3)	0.65
BMI, kg/m^2	29.1 (7.0)	29.8 (7.7)	30.8 (8.6)	29.0 (7.6)	0.06
Race, n (%)					0.67
Caucasian	85 (42.7)	89 (44.7)	82 (41.6)	81 (40.7)	
African American	69 (34.7)	81 (40.7)	86 (43.7)	86 (43.2)	
Hispanic	31 (15.6)	18 (9.0)	21 (10.7)	18 (9.0)	
Other	14 (7.0)	11 (5.5)	8 (4.1)	14 (7.0)	
Blood pressure, mmHg					
Systolic BP	140.1(26.3)	149.9 (29.4)	151.6 (26.4)	150.3 (31.7)	<0.001
Diastolic BP	73.4 (14.9)	80.0 (17.8)	78.6 (14.9)	80.9 (18.2)	<0.001
Cause of renal failure, n (%)					0.23
Diabetes mellitus	81 (40.7)	87 (43.7)	100 (50.8)	91 (45.8)	
Hypertension	68 (34.2)	61 (30.7)	61 (31.0)	68 (34.2)	
Glomerulonephritis	22 (11.1)	33 (16.6)	16 (8.1)	25 (12.6)	
Other	28 (14.1)	18 (9.0)	20 (10.2)	15 (7.5)	
Initial vascular access, n (%)					0.76
Fistula	131 (65.8)	126 (63.3)	124 (62.9)	133 (66.8)	
Graft	38 (19.1)	43 (21.6)	39 (19.8)	43 (21.6)	
Catheter	30 (15.1)	30 (15.1)	34 (17.3)	23 (11.6)	
Comorbidities, n (%)					
Diabetes	102 (51.3)	119 (59.8)	131 (66.5)	124 (62.3)	0.02
Hypertension	95 (47.8)	96 (48.2)	93 (47.2)	88 (44.2)	0.85
Cardiovascular	54 (27.1)	59 (29.6)	64 (32.5)	71 (35.7)	0.29

	Quartile 1 n=199	Quartile 2 n=199	Quartile 3 n=197	Quartile 4 n=199	P value
disease					
Laboratory tests					
Hemoglobin, g/dL	11.3 (1.3)	11.0 (1.2)	10.8 (1.2)	10.7 (1.3)	<0.001
WBC, $x10^{-3}$ per mm^3	7.1 (2.6)	6.7 (2.3)	6.8 (2.3)	6.3 (2.3)	0.016
Platelet count, $x10^{-3}$ per mm^3	241.5 (85.4)	225.3 (69.3)	221.2 (86.9)	209.2 (70.2)	<0.001
RDW, %	14.6 (1.2)	15.0 (1.4)	15.2 (1.7)	15.7 (1.7)	<0.001
Albumin, g/dL	3.9 (0.4)	3.9 (0.4)	3.9 (0.4)	3.9 (0.4)	0.55
Glucose, mg/dL	151.3 (65.0)	166.4 (86.9)	177.0 (98.0)	156.2 (72.3)	0.089
HbA1C, %	6.5 (1.4)	6.6 (1.4)	7.0 (1.5)	6.4 (1.3)	0.009
Calcium, mg/dL	9.2 (0.6)	9.3 (0.6)	9.2(0.6)	9.1 (0.8)	0.12
Phosphate, mg/dL	4.8 (1.4)	5.1 (1.4)	5.3 (1.6)	5.3 (1.7)	<0.001
PTH, pg/mL	355.2 (185.0, 440.5)	369.0 (242.0, 526.0)	347.0 (230.0, 501.0)	335.0 (213.5, 492.5)	0.054
Total cholesterol, mg/dL	148.3 (40.8)	145.7 (33.1)	139.4 (37.8)	134.2 (39.1)	0.015
LDL, mg/dL	80.8 (34.3)	76.2 (27.9)	71.8 (32.7)	69.5 (30.1)	0.027
HDL, mg/dL	40.4 (12.3)	40.4 (11.8)	40.1 (12.5)	40.6 (14.3)	0.99
Serum ferritin, ng/mL	729.0 (456.0, 963.5)	739.0 (528.5, 915.0)	758.0 (492.0, 962.0)	732.0 (471.5, 914.5)	0.40
Iron, ng/mL	72.6 (32.1)	70.0 (26.3)	71.2 (29.4)	62.6 (27.3)	0.0027
Iron saturation, ng/mL	32.5 (14.0)	31.9 (11.9)	31.8 (11.7)	28.1 (11.7)	0.0013
TIBC, mcg/dL	226.6 (42.7)	222.4 (36.5)	224.4 (42.6)	226.3 (47.5)	0.74
UIBC, mcg/dL	154.0(48.4)	152.0 (38.6)	153.1 (40.1)	163.8 (46.9)	0.026
LDH, IU/L	168.5 (37.1)	171.4 (42.2)	184.8 (59.3)	190.0 (50.0)	<0.001
eKt/V	1.4 (0.2)	1.4 (0.3)	1.4 (0.3)	1.4 (0.2)	0.80
URR	74.2 (5.6)	73.9 (5.9)	73.3 (5.7)	73.6 (5.3)	0.47
TBW	41.1 (8.4)	41.8 (8.8)	42.9 (9.5)	41.3 (9.0)	0.18
Dialysis duration, hrs/wk	9.9 (2.5)	9.8 (2.5)	10.2 (2.6)	9.7 (2.3)	0.19
Average EPO dose, unit	2979.6 (2641.8)	3445.0 (2890.9)	4115.1 (4095.6)	5261.4 (4870.4)	<0.001

	Quartile 1 n=199	Quartile 2 n=199	Quartile 3 n=197	Quartile 4 n=199	P value
Ultrafiltration volume, L	2.4 (1.4)	2.6 (1.5)	2.9 (1.6)	3.1 (1.6)	<0.001
Dialysis vintage, months	28.6 (13.8, 57.8)	38.2 (18.5, 81.8)	34.8 (17.8, 67.0)	48.8 (23.3, 81.8)	0.027

Legend: Values for continuous variables are presented as mean (standard deviation), median (interquartile range), or range. Abbreviations: BMI, body mass index; BP, blood pressure; WBC, white blood cell; RDW, red blood cell distribution width; HbA1C, hemoglobin A1C; PTH, parathyroid hormone; LDL, low-density lipoprotein; HDL, high-density lipoprotein; TIBC, total iron binding capacity; UIBC, unsaturated iron binding capacity; LDH, lactate dehydrogenase; eKt/V, equilibrated Kt/V; URR, urea reduction ratio; TBW, total body water (Watson formula), average EPO (erythropoietin) dosage were calculated as total EPO dosage/using times one month before baseline time for each patient.

Baseline ET-1 and Mortality

During a median follow-up period of 27.8 months, 253 individuals (31.9%) died. The incidence of death was 65.1, 63.9, 68.4, and 79.3 per 100 person-years for the first, second, third, and fourth quartile of baseline ET-1, respectively (Table 2 and Figure 1). Higher baseline ET-1 levels were associated with a higher risk of death in both unadjusted and adjusted analyses. In the fully adjusted model including clinical characteristics, dialysis adequacy, access, dialysis vintage, and laboratory values, each 1 unit SD increase in log-ET was associated with a 1.46-fold increased risk of death (HR 1.46, 95% CI 1.27 – 1.68). The association between ET-1 and mortality remained consistent in different clinical subgroup analyses (Figure 2) and was accentuated in patients with dialysis vintage more than 12 months (compared to less than 12 months, P for interaction = 0.021) and in those with vs. without prevalent CVD (P for interaction = 0.002).

Table 2. Risk of death according to plasma endothelin-1 levels

Plasma endothelin-1, ng/mL	Quartile 1 0.26-1.56	Quartile 2 1.57-2.02	Quartile 3 2.02-2.71	Quartile 4 2.71-14.51	*P* value for trend	Continuous endothelin-1, per 1 SD log-ET	*P* value
Number of participants	199	199	197	199	-	-	-
Death incidence rate, per 100-person years)	10.7	12.3	16.6	24.1	-	-	-
Hazard ratio (95% CI)							
Model 1: unadjusted	Reference	1.15 (0.77 to 1.72)	1.53 (1.05 to 2.24)	2.20 (1.54 to 3.15)	<0.001	1.43 (1.26 to 1.61)	<0.001
Model 2: Adjusted for age, race, sex, BMI, SBP, diabetes as cause of ESRD, hemodialysis access, vintage, history of CVD	Reference	1.45 (0.96 to 2.18)	1.90 (1.28 to 2.82)	3.15 (2.15 to 4.60)	<0.001	1.59 (1.39 to 1.81)	<0.001
Model 3: Model 2 + albumin, ferritin, eKt/V	Reference	1.51 (1.00 to 2.27)	1.98 (1.34 to 2.94)	3.08 (2.11 to 4.51)	<0.001	1.55 (1.36 to 1.76)	<0.001
Model 4: Model 3 + iron, TIBC, LDH, hemoglobin, WBC, platelet count, RDW, PTH, phosphate, calcium	Reference	1.39 (0.92 to 2.11)	1.82 (1.21 to 2.73)	2.52 (1.68 to 3.79)	<0.001	1.46 (1.27 to 1.68)	<0.001

Abbreviations: CVD, cardiovascular disease; BMI, body mass index; SBP, systolic blood pressure; ESRD, end-stage renal disease; eKt/V, equilibrated Kt/V; TIBC, total iron binding capacity; LDH, lactate dehydrogenase; RDW, red blood cell distribution width; PTH, parathyroid hormone

Fig. 1. Kaplan-Meier curves of the four ET-1 categories (all-cause mortality)

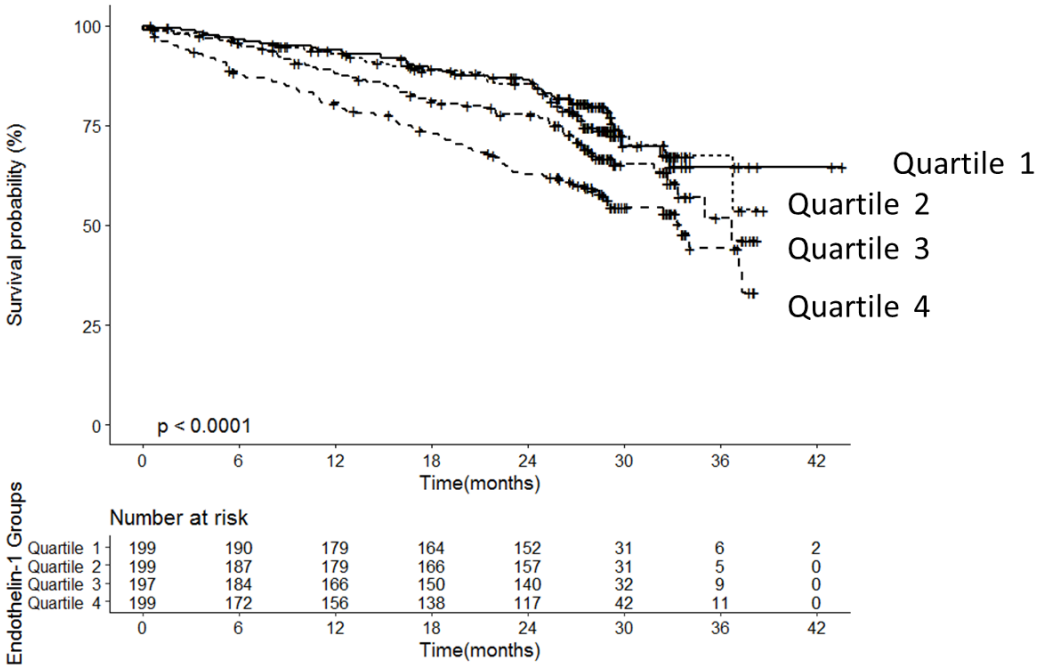
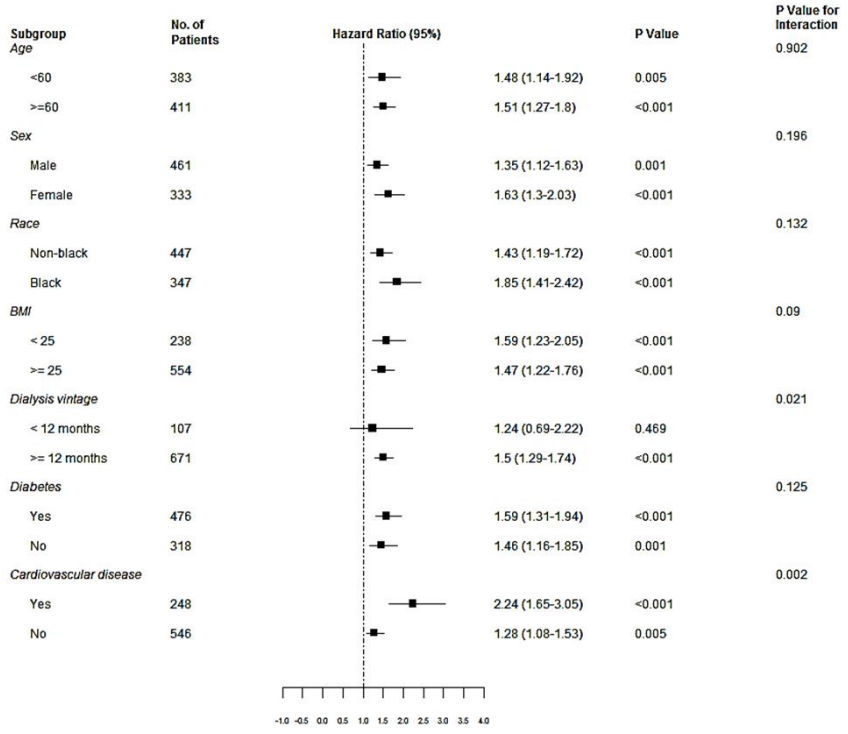


Fig. 2. Subgroup analysis of the association between log-ET and HD patients' all-cause mortality



* For BMI, the missing value(NA) was 2; for vintage, the missing value(NA) was 16

Baseline ET-1 and hospitalization

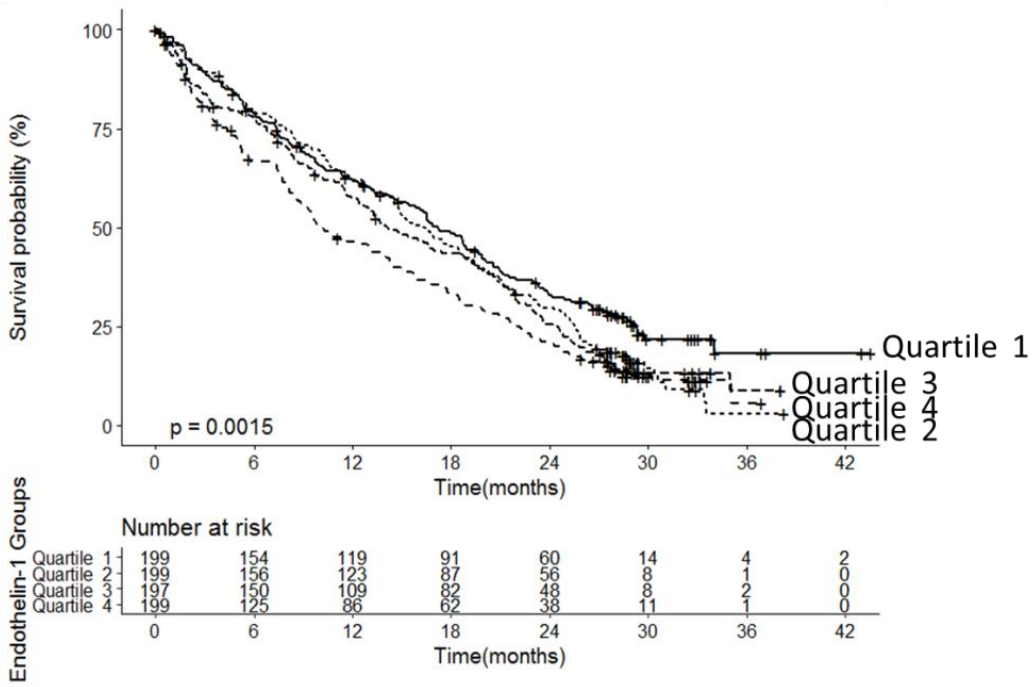
During a median follow-up period of 27.8 months, 643 individuals (81.0%) were hospitalized at least once. Higher baseline ET-1 levels were associated with a higher risk of hospitalization in both unadjusted and adjusted analyses (Table 3 and Figure 3). In the fully adjusted model including clinical characteristics, dialysis adequacy, access, vintage, and laboratory values, each 1 SD increase in log-ET was associated with a 1.13-fold increased risk of hospitalization (HR 1.16, 95% CI 1.04 – 1.23).

Table 3. Risk of hospitalization according to plasma endothelin-1 levels

Plasma endothelin-1, ng/mL	Quartile 1 0.26-1.56	Quartile 2 1.57-2.02	Quartile 3 2.02-2.71	Quartile 4 2.71-14.51	*P* value for trend	Continuous endothelin-1, per 1 SD log-ET	*P* value
Number of participants	199	199	197	199	-	-	-
Incidence rate, per 100-person years)	53.4	64.0	68.4	80.6	-	-	-
Hazard ratio (95% CI)							
Model 1: unadjusted	Reference	1.22 (0.97 to 1.52)	1.31 (1.04 to 1.63)	1.55 (1.24 to 1.94)	<0.001	1.15 (1.06 to 1.24)	<0.001
Model 2: Adjusted for age, race, sex, BMI, SBP, diabetes as cause of ESRD, hemodialysis access, vintage, history of CVD	Reference	1.26 (1.00 to 1.59)	1.32 (1.05 to 1.67)	1.67 (1.32 to 2.11)	<0.001	1.18 (1.08 to 1.28)	<0.001
Model 3: Model 2 + albumin, ferritin, eKt/V	Reference	1.26 (1.00 to 1.59)	1.33 (1.06 to 1.68)	1.70 (1.34 to 2.14)	0.001	1.18 (1.09 to 1.28)	0.008
Model 4: Model 3 + iron, TIBC, LDH, hemoglobin, WBC, platelet count, RDW, PTH, phosphate, calcium	Reference	1.24 (0.98 to 1.57)	1.25 (0.98 to 1.58)	1.52 (1.18 to 1.95)	0.002	1.13 (1.04 to 1.23)	0.005

Abbreviations: CVD, cardiovascular disease; BMI, body mass index; SBP, systolic blood pressure; ESRD, end-stage renal disease; eKt/V, equilibrated Kt/V; TIBC, total iron binding capacity; LDH, lactate dehydrogenase; RDW, red blood cell distribution width; PTH, parathyroid hormone

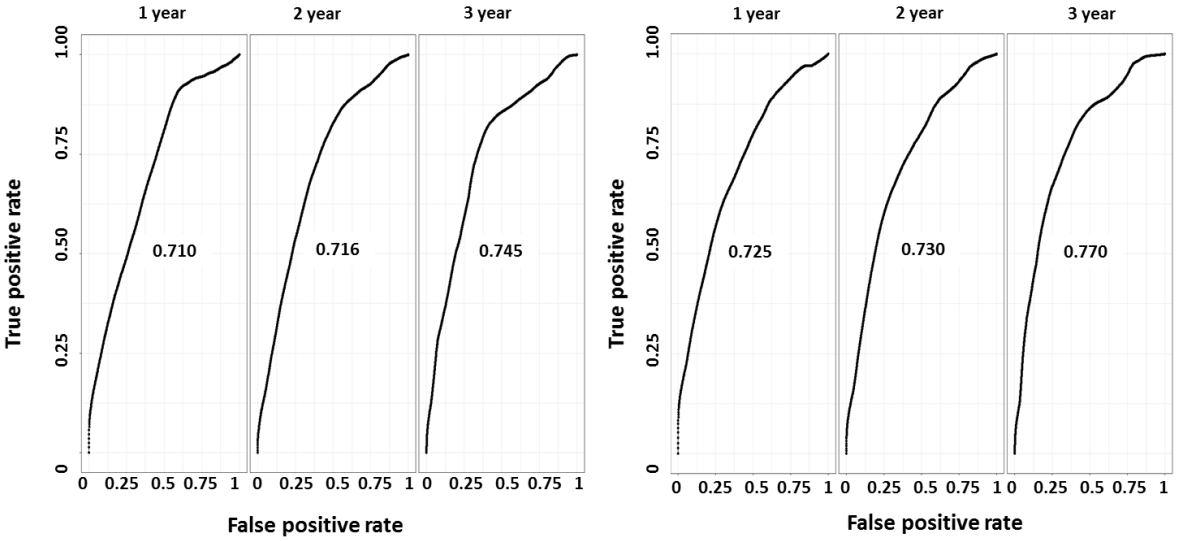
Fig. 3. Kaplan-Meier curves of the four ET-1 categories for hospitalization



Assessment of Discrimination in Survival Analysis

We next compared whether ET-1 improved risk prediction for mortality or cardiovascular disease hospitalization. For all-cause mortality, the Harrell's C-statistic of the fully adjusted model increased from 0.723 (SE 0.019) to 0.738 (SE 0.019) after addition of log-ET (P<0.001). For hospitalization, the Harrell's C-statistic of the fully adjusted model increased from 0.608 (SE 0.013) to 0.613 (SE 0.013) after addition of log-ET (P=0.005). The time-dependent ROC curves in a fully adjusted model for mortality and hospitalization with log-ET both demonstrated more accurate prediction than the model without log-ET in each year during the follow-up. (Figure 4 and 5).

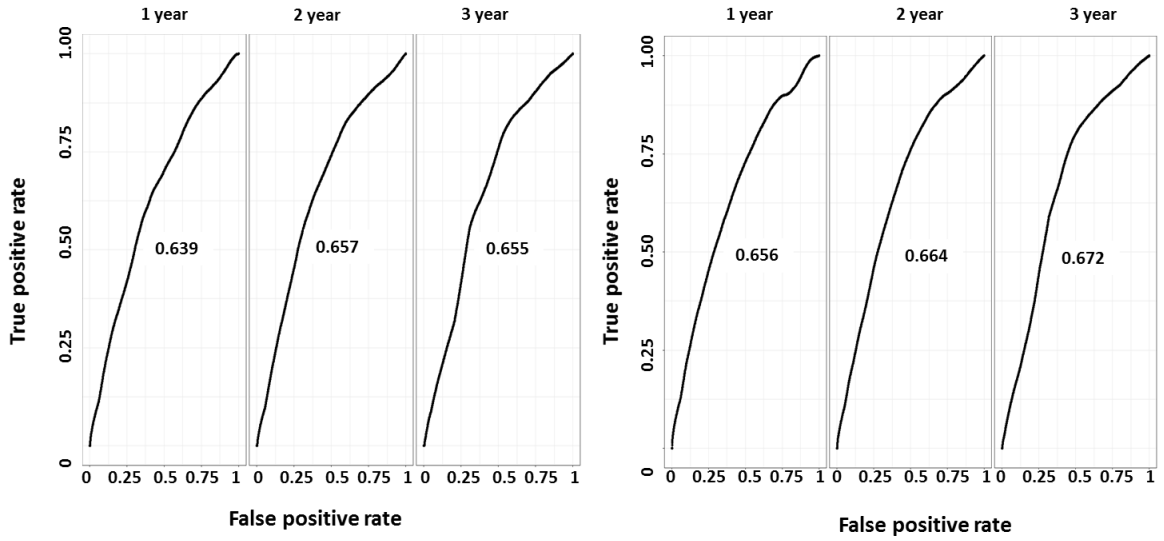
Fig. 4. Time-dependent ROC curves for all-cause mortality



a. ROC curves for model 4 without log-ET in 1, 2 and 3year

b. ROC curves for model 4 with log-ET in year 1, 2 and 3

Fig. 5. Time-dependent ROC curves for hospitalization



a. ROC curves for model 4 without log-ET in 1, 2 and 3year

b. ROC curves for model 4 with log-ET in year 1, 2 and 3

Discussion

We found that higher baseline levels of plasma ET-1 were independently associated with both an increased risk of death as well as increased risk of cardiovascular disease hospitalization among maintenance hemodialysis patients. Furthermore, the addition of ET-1 to the risk prediction model significantly improved prediction for both outcomes as judged by the increase in c statistic.

ET-1 is a potent vasoactive substance which is primarily released by endothelial and vascular smooth muscle cells under conditions of inflammation, vascular stress, and hypoxia. (22-24) The effects of ET-1 are mediated through two receptor subtypes, ET_A and ET_B. While sustained vasoconstrictive responses are mediated by receptor ET_A, ET_B stimulates endothelial-dependent relaxation. (25, 26) Upregulation of ET-1 has been implicated in distinct pathways in the pathogenesis of the cardiovascular disease. (22, 23) Several epidemiological studies have demonstrated the associations of ET-1 with the incidence of congestive heart failure (27, 28), pulmonary hypertension (27, 29), and CKD(30). ET-1 has also been shown to be an independent predictor of death and cardiovascular outcomes in disease states such as heart failure(31, 32), pulmonary hypertension(29), and coronary artery disease(33). In patients on hemodialysis, smaller studies have shown associations of ET-1 levels with ischemic heart disease(8) and other surrogate cardiovascular end-points such as intradialytic hypertension(34-36), endothelial dysfunction(37) and incidence of atherosclerosis(38). However, less is known about ET-1 as a predictor of hard outcomes in the hemodialysis population.

ESRD is a unique clinical setting notable for exceedingly high rates of morbidity and mortality. Indeed, the risk of cardiovascular disease and mortality in ESRD patients is 5 to

30 times higher than in the general population(39). There are several reasons for ET-1 to be elevated in ESRD. Reduced catabolism and clearance by the failing kidneys may contribute, though animal studies have shown that lung and liver metabolism are also important determinants of ET-1 clearance. (40) Certain clinical hallmarks of ESRD have been implicated in the increased production of ET-1 by endothelial cells. These include endothelial injury and shear stress(41), as well as academia (42, 43) and venous congestion(44). In animal studies, ET_B receptors, which have been implicated in the clearance of circulating ET-1(45), were found to be down-regulated in the endothelium in a uremic milieu(26). Increases in ET-1 may be maladaptive in ESRD and contribute to cardiovascular disease pathogenesis and mortality from its vasoactive properties and/or its mitogenic effects on vascular smooth muscle cells. ET-1 contributes to endothelial dysfunction and atherosclerosis, and the higher ET-1 may lead to atherosclerosis in HD patients. (46, 47) Also, arterial stiffness is an independent risk factor for mortality in HD patients, and ET-1 is the major hormone regulating vascular tension in vivo. (48)

Although our findings cannot show causality between ET-1 and adverse outcomes in ESRD, they raise the question whether ET-1 antagonism could be promising therapeutic approach in treating ESRD patients, if ET-1 is indeed pathogenic in ESRD. Endothelin receptor antagonists have long been discussed as potential therapeutic targets to improve clinical outcomes in cardiovascular disease. Initial trials showed beneficial effects of endothelin receptor blockade through attenuation of inflammation and fibrotic remodeling(49). However, ET_A blockade has not yet emerged in daily clinical practice due to the observed, severe side effects such as increased fluid retention (12). The disappointing results of ET-1 antagonism in heart failure are thought to be due to side

effects, particularly volume retention. It is worth highlighting that in ESRD, volume regulation is primarily through the hemodialysis procedure; ET-1 antagonists would therefore not be expected to lead to volume retention in most ESRD patients, who are typically oligoanuric.

There are several important limitations to our study. Cardiovascular hospitalization endpoints were not adjudicated, so misclassification is possible. We attempted to control for common potential confounding variables, but residual confounding may still exist. Patients consented and enrolled in this biorepository are from a single large dialysis organization (but with sites across the country); as a result, the findings may not be generalizable to the overall hemodialysis population. Whether ET-1 levels were higher because of reduced clearance from ESRD or because of increased production is not clear.

Moreover, a causal relationship between ET-1 and hard outcomes cannot be established from this study. In our study, ET-1 levels were obtained as a single time-point measurement before hemodialysis; thus, we could not demonstrate its response to various intradialytic pathophysiologic events. Moreover, the relationship between ET-1 and blood pressure needs further investigation, as prior studies have demonstrated associations between intradialytic hypertension and dynamic changes in ET-1 levels that occur during and after hemodialysis(35, 36, 50, 51).

In conclusion, higher levels of plasma ET-1 are independently associated with an increased risk of mortality and hospitalization in ESRD patients on maintenance hemodialysis. Our findings point towards a potential role for ET-1 antagonists as a

pharmacological intervention in ESRD which should be addressed in future interventional trials.

Paper 2: Risk factors for elevated endothelin-1 level in hemodialysis patients

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Abstract

ET-1 increases strikingly among hemodialysis patients, but the risk factor and the clinical relevance for elevated ET-1 are not clear. Also the association between erythropoietin (EPO)-induced hypertension and ET-1 is not well understood. We measured plasma ET-1 levels in a cohort of 794 individuals with prevalent ESRD treated with maintenance hemodialysis. By best subset regression, diastolic BP (per 10 mmHg, coefficient 0.039, P<0.001), hemoglobin concentration (per 1 g/dL, coefficient -0.045, P<0.001), platelet count (per 100 10^{-3}/ mm^3, coefficient -1.01, P<0.001), RDW (per 1%, coefficient 0.052, P<0.001), iron saturation (per 10 ng/mL, coefficient -0.045, P<0.001), LDH (per 100 IU/L, coefficient 0.126, P<0.001), and ultrafiltration volume (per 1 L, coefficient 0.044, P<0.001) were the most important clinical factors associated with ET-1 level. Among 794 patients, 685 (86.3%) patients received EPO therapy four weeks before the blood draw date. Compared with the patients who did not receive EPO therapy, the ET-1 level (P=0.032) and systolic blood pressure (BP) (P<0.001) of patients who received EPO treatment were higher. By mediation analysis, the association of ET-1 with higher systolic blood pressure was only mildly mediated by EPO treatment (0.08%, 95%CI 0.002%-0.44%, P=0.040). Among the participants who received EPO, log-transformed average EPO dosage/week did not show association with systolic BP or diastolic BP (P>0.05).

Introduction

ET-1 is mainly synthesized by endothelial cells, consistent with its role as a potent vasoconstrictor. Additional sites of production are the heart, brain, and kidney. ET-1 is essential to the function of various organs and metabolic processes, including vascular homeostasis, inflammation, and cell growth (9). Elevated ET-1 has been implicated in a variety of conditions including hypertension, congestive heart failure, inflammation and tumor (19, 46, 47, 52, 53). End-stage renal disease (ESRD) patients are a special population at very high risk of CVD and share many key pathophysiologic features associated with high levels of ET-1. ESRD patients were reported to have high levels of ET-1 compared with healthy controls, patients with hypertension, pulmonary hypertension or chronic kidney disease. (29, 54) Our previous study has shown that higher plasma ET-1 appears to be associated with adverse events in hemodialysis patients independent of previously described risk factors.

The regulation of ET-1 is very complex and not well understood, especially under disease conditions. ET-1 can be stored in cells and released when stimulated by many factors including transforming growth factor-β (TGF-β), interleukin-1 (IL-1), angiotensin II, adhesion molecules and growth factors. On the other hand, nitric oxide (NO), prostacyclin and atrial natriuretic peptide inhibit ET-1 synthesis(55). Plasma ET-1 levels are increased in CKD and end-stage renal disease compared with healthy individuals (56, 57). Since ET-1 has a pathogenic role in many kidney diseases and cardiovascular diseases, we were interested in identifying the risk factors for HD patients to have a higher level of ET-1. Previous studies have shown that hemodialysis patients exhibited the most strikingly elevated levels of ET-1, but the risk factors for elevated ET-1 are not clear(34, 58).

Erythropoietin (EPO) is widely used in HD patients for the treatment of anemia. Increased blood pressure is a common adverse effect of EPO therapy (59). ET-1 has been implicated as a major mediator of the association between EPO and hypertension, but results are inconsistent and most of which are from pre-clinical studies(60). We, therefore, sought to investigate the association between EPO treatment and ET-1 levels in patients undergoing hemodialysis.

Methods

Study design and population

Plasma samples and data were obtained from a cohort study and biorepository assembled by DaVita Clinical Research (DCR). Between May 2011 and October 2013, 4,028 individuals with prevalent end stage renal disease treated with hemodialysis provided written informed consent to participate in a longitudinal cohort study with four quarterly collections of biospecimens along with baseline and clinical data collection. Inclusion criteria included age $\geq$ 18 years and treatment for ESRD with hemodialysis. Subjects were excluded for Hgb < 8.0 g/dL, pregnancy, or any physical, mental, or medical condition which prohibited the ability to provide written informed consent. Clinical data and hemodialysis treatment data were collected and maintained for each study subject in the normal course of care using the DaVita electronic medical record. The last follow-up time was December 31, 2014. Anonymized and de-identified data for the study were transferred from the electronic data system into a separate trial database for research use. Samples and data were distributed equally to four academic medical centers. Each center responded successfully to a competitive request for applications of biospecimens and clinical data. All

patients provided written informed consent prior to the initiation of sample collection. The study met minimal risk criteria as set forth and defined at 21 CFR 56.111.

Biospecimen collection and storage

Pre-dialysis blood samples were collected and processed according to a standardized protocol, which included shipping on refrigerated packs on the same day as collection. Specimens were shipped to DaVita Labs for processing, aliquoting, and storage at -80°C. Specimens were rejected, and re-collection requested for any specimen with cause for rejection (e.g., unspun tubes, insufficient volume, or thawed specimens). Specimens received > 48h from the time of collection were also rejected and re-collected. Samples were distributed frozen at -80°C across four academic medical centers from the DaVita Lab.

Measurement of Endothelin-1 (ET-1)

Pre-dialysis plasma levels of ET-1 in baseline samples were measured in duplicates using a commercially available ELISA kit (Quantikine Human ET-1 Immunoassay PDET100; R&D Systems, Minneapolis, MN). We assessed the inter-assay coefficient of variation using 42 blind splits replicate plasma samples obtained from ESRD patients recruited at Brigham and Women's Hospital. The mean inter-assay CV from blind split replicates was 7.3%.

Assessment of Covariates

We collected data on demographics, clinical conditions, hemodialysis prescription data, comorbidities, and laboratory data from DCR. Laboratory tests were measured on blood samples collected pre-dialysis, except for post-dialysis BUN. Ultrafiltration volume was assessed by weight difference before vs. after the hemodialysis. In DaVita electronic

medical record, all the erythropoietin (EPO) was recorded as "Epogen" without specific drug names. For the EPO dosage and blood pressure, we collected all the data 4 weeks prior to the blood draw date. The other baseline covariate data were chosen according to the collection time point closest to the first study sample blood draw date.

Analytic approach

All analyses were performed in R version 3.4.2. We expressed continuous variables as means (standard deviations) or medians (IQR, 25th and 75th percentiles). To find the clinical and laboratory factors associated ET-1 levels, we used natural log-transformed plasma ET-1 as the dependent variable in univariable linear regression models (with significance set at $P < 0.05/24 = 0.002$) and a multivariable linear model to identify independent factors. We also explored automated regression models including stepwise selection and best subset regression to find the best predictors for plasma ET-1. For stepwise selection, the final model was selected by the smallest Akaike information criterion (AIC) value. We did not restrict any predictors and the number of predictors during the model selection procedure. We used two statistical methods that came with the results from best subset regression: Mallow' Cp and Bayesian Information Criteria. Multicollinearity was tested by a variance inflation factor (VIF), and homoscedasticity was confirmed by Breusch-Pagan test. We calculated the average erythropoietin (EPO) dosage per week and average EPO dosage per prescription for each patient one month prior to blood draw time. Since the EPO dosage distribution was skewed, for the patients who used EPO, we used natural log-transformed average weekly EPO dosage for analyzing. We used mediation analysis to estimate how much contribution of the mediator (ET-1) to EPO-

induced hypertension. The mediation analysis used the R package "mediation," and the number of simulations was set to 1000. Two linear regression models were used in the mediation analysis as instructed in the package: one mediator model which taken log-transformed ET-1 as the independent variable; the second model is outcome regression model which taken blood pressure as the independent variable. The average direct effects (ADE) of EPO on blood pressure and the average causal mediation effects of EPO on blood pressure trough ET-1 (ACME) were calculated from the two linear models. Complete data sets on variables for outcome analyses were available in 97% of the cohort. We found no appreciable difference in results with multiple imputations by R package "mice" and therefore missing data were not imputed in the primary analysis. All statistical tests were two-sided, and P values < 0.05 were considered statistically significant for time to event analyses.

Results

Study population characteristics

A total of 792 participants were included in this study. At baseline, mean age was 60.0 $\pm$ 13.8 years, median dialysis vintage was 37.2 months (interquartile range (25-75th percentile, IQR), 18.1 to 72.6 months), and 58% were men. The most common reasons for ESRD were diabetes mellitus (45.0%), hypertension (31.9%), and other glomerular diseases (12.0%). The median plasma ET-1 level was 2.02 (IQR, 1.57 – 2.71) pg/mL. (Table 1).

Table 1. Baseline characteristics of participants

	All participants (n=792)
Plasma endothelin-1, pg/mL	2.02 (1.56, 2.71)
Age, years	60.1 (13.8)
Male, n (%)	461 (58.1)
BMI, kg/m²	29.7 (7.7)
Race, n (%)	
Caucasian	337 (42.4)
African American	322 (40.6)
Hispanic	88 (11.1)
Other	47 (5.9)
Blood pressure, mmHg	
Systolic BP	148.0 (28.9)
Diastolic BP	78.2 (16.7)
Cause of renal failure, n (%)	
Diabetes mellitus	359 (45.2)
Hypertension	258 (32.5)
Glomerulonephritis	96 (12.1)
Other	81 (10.2)
Initial vascular access, n (%)	
Fistula	514 (64.7)
Graft	163 (20.5)
Catheter	117 (14.7)
Comorbidities, n (%)	
Diabetes	476 (60.0)
Hypertension	372 (46.9)
Cardiovascular disease	248 (31.2)
Laboratory tests	
Hemoglobin, g/dL	10.9 (1.3)
Hematocrit	35.4 (3.7)
WBC, $x10^{-3}$ per mm^3	6.7 (2.4)
Platelet count, $x10^{-3}$ per mm^3	224.3(79.1)
RDW, %	15.1 (1.6)
Albumin, g/dL	3. 9 (0.4)
Glucose, mg/dL	163.5 (82.9)
HbA1C, %	6.6 (1.4)
Calcium, mg/dL	9.2 (0.7)
Phosphate, mg/dL	5.1 (1.5)
PTH, pg/mL	334.5 (216.5, 492.5)
Total cholesterol, mg/dL	141.7 (38.2)
LDL, mg/dL	74.4 (31.6)
HDL, mg/dL	40.4 (12.8)
Serum ferritin, ng/mL	738.5 (488.0, 932.8)

	All participants (n=792)
Iron, ng/mL	69.1 (29.1)
Iron saturation, ng/mL	31.1 (12.4)
TIBC, mcg/dL	224.9 (42.5)
UIBC, mcg/dL	155.7 (43.9)
LDH, IU/L	178.6 (48.6)
eKt/V	1.4 (0.2)
URR	73.7 (5.6)
TBW	41.8 (8.9)
Dialysis duration, hrs/wk	9.9 (2.5)
Average EPO dose per time, unit	3949 (3757.4)
Ultrafiltration volume, L	2.7 (1.5)
Dialysis vintage, months	37.2 (18.1, 72.6)

Legend: Values for continuous variables are presented as mean (standard deviation), median (interquartile range), or range. Abbreviations: BMI, body mass index; BP, blood pressure; WBC, white blood cell; RDW, red blood cell distribution width; HbA1C, hemoglobin A1C; PTH, parathyroid hormone; LDL, low-density lipoprotein; HDL, high-density lipoprotein; TIBC, total iron binding capacity; UIBC, unsaturated iron binding capacity; LDH, lactate dehydrogenase; eKt/V, equilibrated Kt/V; URR, urea reduction ratio; TBW, total body water (Watson formula), average EPO (erythropoietin) dosage were calculated as total EPO dosage/using times one month before baseline time for each patient.

Factors associated with ET-1 levels

Cross-sectional analyses of clinical and laboratory factors with ET-1 levels are shown in Table 2. In univariable models, clinical variables that correlated with higher ET-1 levels included lower age, hemoglobin, platelet count, and iron saturation; and higher diastolic blood pressure, RDW, serum iron, LDH, and ultrafiltration volume (all $P<0.001$). In the multivariable model, hemoglobin, platelet count, RDW, LDH, and ultrafiltration volume were the strongest independent factors associated with ET-1 levels (all $P<0.001$). The β coefficients and P values are from regression models of natural-log transformed plasma endothelin-1 levels as the dependent variable. (100 x β) can be interpreted as the approximate percentage difference in plasma endothelin-1 levels.

Table 2. Clinical and laboratory factors associated with log-ET

Variable	Univariate analysis		Multivariable analysis		Stepwise selection	
	Coefficient	*P* value	Coefficient	*P* value	Coefficient	*P* value
Age, per 10 years	-0.039	<0.001	-0.026	0.038	-0.026	0.032
Sex, male vs. female	-0.02	0.526	-0.058	0.087	-0.055	0.082
BMI, per 1 kg/m²	-0.0009	0.636	-0.005	0.017	-0.005	0.012
Systolic BP, per 10 mmHg	0.016	0.004	0.001	0.11	0.011	0.095
Diastolic BP, per 10 mmHg	0.035	<0.001	0.002	0.148	0.019	0.122
Hemoglobin, per 1 g/dL	-0.057	<0.001	-0.047	<0.001	-0.05	<0.001
WBC, per 10^{-3}/ mm³	-0.017	0.012	-0.003	0.646	-	-
Platelet count, per 100 10^{-3}/ mm³	-0.075	<0.001	-0.099	<0.001	-0.01	<0.001
RDW, per 1%	0.074	<0.001	0.045	<0.001	0.05	<0.001
Albumin, per 1 g/dL	-0.041	0.313	0.013	0.767	-	-
Calcium, per 1 mg/dL	-0.006	0.798	-0.005	0.83	-	-
Phosphate, per 1 mg/dL	0.032	0.002	0.022	0.037	0.022	0.031
PTH, per 10 pg/mL	0.0004	0.477	-0.0008	0.115	-0.0008	0.097
Iron, per 10 ng/mL	0.02	<0.001	0.104	0.052	0.024	0.04
Iron saturation, per 10 ng/mL	-0.052	<0.001	-0.13	0.028	-0.1	<0.001
TIBC, per 100 mcg/dL	0.004	0.91	-0.706	0.139	-	-
UIBC, per 100 mcg/dL	0.094	0.009	0.77	0.164	-	-
LDH, per 100 IU/L	0.018	<0.001	0.132	<0.001	0.129	<0.001
eKdt/V	0.01	0.877	-0.096	0.552	-	-
URR	-0.001	0.626	0.003	0.619	-	-
Hrs/Week Treated, per 10 hr/wk	0.002	0.795	0.037	0.634	-	-
Average EPO dose per time, per 1000 Units	0.023	<0.001	0.005	0.298	-	-
Ultrafiltration volume, per 1 L	0.052	<0.001	0.051	<0.001	0.049	<0.001
Dialysis vintage, per 1 year	0.009	0.02	0.006	0.04	0.007	0.027

Legend: Values for continuous variables are presented as mean (standard deviation), median (interquartile range), or range. Abbreviations: BMI, body mass index; BP, blood pressure; WBC, white blood cell; RDW, red blood cell distribution width; HbA1C, hemoglobin A1C; PTH, parathyroid hormone; LDL, low-density lipoprotein; HDL, high-density lipoprotein; TIBC, total

iron binding capacity; UIBC, unsaturated iron binding capacity; LDH, lactate dehydrogenase; eKt/V, equilibrated Kt/V; URR, urea reduction ratio; TBW, total body water (Watson formula), average EPO (erythropoietin) dosage were calculated as total EPO dosage/using times one month before baseline time for each patient.

By the lowest Mallow's Cp, 15 variables were the best fit in the linear regression model. After checking for collinearity and homoscedasticity, there was collinearity between iron saturation and iron. Iron was excluded in the final linear model. By the lowest Bayesian Information Criteria, a seven-feature model was appropriate which included diastolic BP, hemoglobin concentration, platelet count, RDW, iron saturation, LDH, and ultrafiltration volume. Results were generally consistent in the stepwise selection and best subset regression models (Table3 and figure 1).

Table 3. Clinical and laboratory factors associated with log-ET by best subset regression

Variable	**Lowest Mallow's Cp**		**Lowest Bayesian Information Criteria**	
	Coefficient	***P* value**	**Coefficient**	***P* value**
Age, per 10 years	-0.026	0.035	-	-
Sex, male vs. female	-0.055	0.085	-	-
BMI, per 1 kg/m²	-0.005	0.015	-	-
Systolic BP, per 10 mmHg	0.021	0.095	-	-
Diastolic BP, per 10 mmHg	0.011	0.111	0.039	<0.001
Hemoglobin, per 1 g/dL	-0.051	<0.001	-0.045	<0.001
WBC, per 10^{-3}/ mm³	-	-	-	-
Platelet count, per 100 10^{-3}/ mm³	-1.033	<0.001	-1.01	<0.001
RDW, per 1%	0.049	<0.001	0.052	<0.001
Albumin, per 1 g/dL	-	-	-	-
Calcium, per 1 mg/dL	-	-	-	-
Phosphate, per 1 mg/dL	0.022	0.034	-	-
PTH, per 10 pg/mL	0.0008	0.093	-	-
Iron, per 10 ng/mL	-	-	-	-
Iron saturation, per 10 ng/mL	-0.081	0.128	-0.045	<0.001
TIBC, per 100 mcg/dL	0.164	0.33	-	-
UIBC, per 100 mcg/dL	-0.148	0.519	-	-
LDH, per 100 IU/L	0.13	<0.001	0.126	<0.001
eKdt/V	-	-	-	-
URR	-	-	-	-
Hrs/Week Treated, per 10 hr/wk	-	-	-	-
Average EPO dose per time, per 1000 Units	-	-	-	-
Ultrafiltration volume, per 1 L	0.048	<0.001	0.044	<0.001
Dialysis vintage, per 1 year	0.007	0.028	-	-

Legend: Values for continuous variables are presented as mean (standard deviation), median (interquartile range), or range. Abbreviations: BMI, body mass index; BP, blood pressure; WBC, white blood cell; RDW, red blood cell distribution width; HbA1C, hemoglobin A1C; PTH, parathyroid hormone; LDL, low-density lipoprotein; HDL, high-density lipoprotein; TIBC, total iron binding capacity; UIBC, unsaturated iron binding capacity; LDH, lactate dehydrogenase; eKt/V, equilibrated Kt/V; URR, urea reduction ratio; TBW, total body water (Watson formula), average EPO (erythropoietin) dosage were calculated as total EPO dosage/using times one month before baseline time for each patient.

Figure 1. a. The Plot of Mallow's Cp in best subset regression

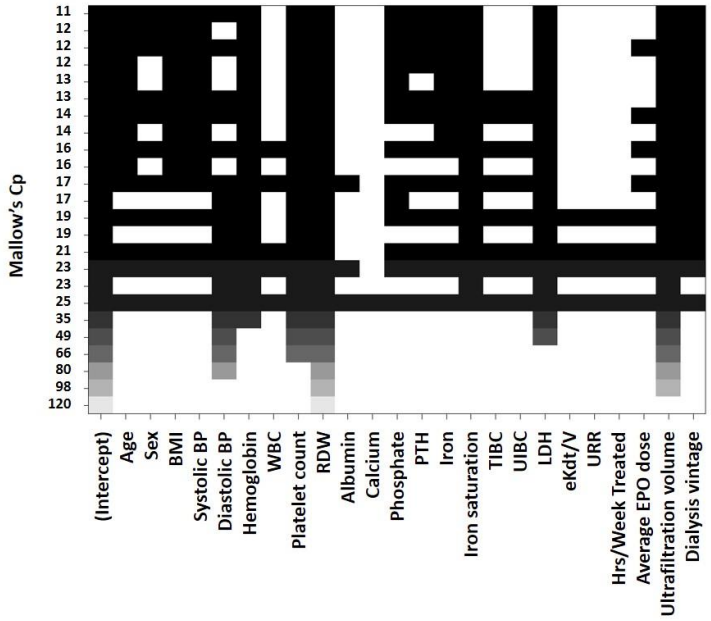


Figure 1. Mallow's Cp estimates the size of the bias that is introduced into the predicted responses by having an underspecified model. By including 15 variables which reached the top in the figure, the Mallow's Cp stops decreasing (from 120 to 11).

Figure 1. b. The plot of Bayesian Information Criteria in best subset regression

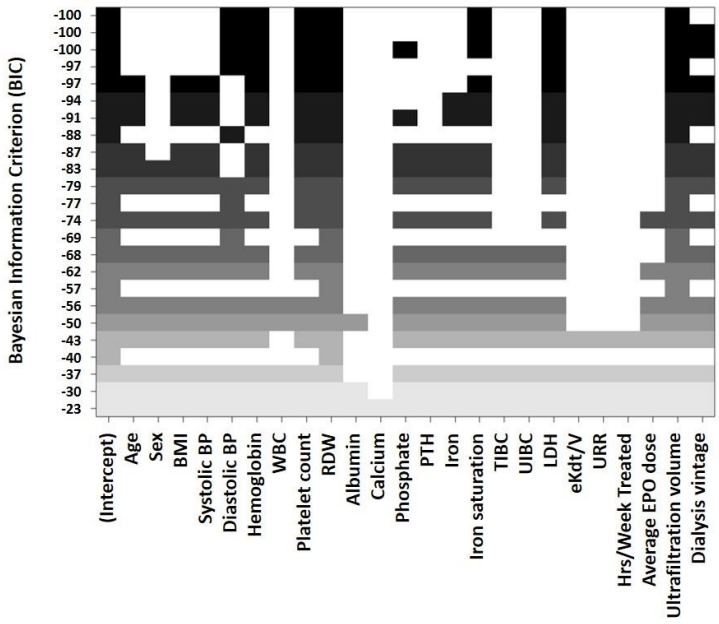


Figure 1. b Lower BIC indicates lower penalty terms. By including seven variables which reached the top, the BIC reached the smallest value (from -23 to -100).

The relationship between EPO usage, ET-1 levels, and blood pressure

Among 794 patients, 685 (86.3%) patients received EPO therapy four weeks before the blood draw date. Table 4 shows results comparing characteristics between patients who did vs. did not receive EPO therapy during the four weeks before the initial blood draw. The patients who did not receive EPO treatment were more likely to be male ($P<0.001$). Compared with the patients who did not receive EPO therapy, the ET-1 level ($P=0.032$) and Systolic BP ($P<0.001$) of patients who received EPO treatment were higher. Diastolic BP was not different between groups ($P=0.462$). The patients who received EPO treatment had lower hemoglobin, hematocrit, iron TIBC and UIBC levels. The patients who received EPO treatment had a higher level of RDW and serum ferritin ($P<0.001$). The patients who received EPO treatment had better dialysis adequacy. (table 4)

Table 4. The characteristics of patients with and without EPO therapy

	EPO therapy (n=685)	Without EPO therapy (n=109)	P value
Plasma endothelin-1, pg/mL	2.32 (1.2)	2.09 (1.0)	0.032
Age, years	60.5 (13.9)	57.3 (13.0)	0.018
Male, n (%)	377 (55.0)	84 (77.1)	<0.001
BMI, kg/m²	29.8 (8.0)	29.0 (6.2)	0.220
Race, n (%)			0.807
Caucasian	286 (41.8)	51 (46.8)	
African American	281 (41.0)	41 (37.6)	
Hispanic	78 (11.4)	10 (9.2)	
Other	40 (5.8)	7 (6.4)	
Blood pressure, mmHg			
Systolic BP	149.1 (28.5)	140.9 (30.3)	0.008
Diastolic BP	78.0 (16.6)	79.3 (17.3)	0.462
Laboratory tests			
Hemoglobin, g/dL	10.7 (1.0)	12.5 (1.7)	<0.001
Hematocrit, %	34.6 (2.8)	40.3 (4.8)	<0.001
WBC, $x10^{-3}$ per mm^3	6.7 (2.4)	7.0 (2.2)	0.160
Platelet count, $x10^{-3}$ per mm^3	224.6 (80.2)	222.2 (72.1)	0.747
RDW, %	15.2 (1.6)	14.7 (1.5)	<0.001
Albumin, g/dL	3. 9 (0.4)	4.0 (0.4)	0.044
Serum ferritin, ng/mL	755.7 (319.0)	572.1 (387.9)	<0.001
Iron, ng/mL	68.0 (28.8)	76.1 (30.1)	0.008
Iron saturation, ng/mL	31.0 (12.3)	31.8 (13.1)	0.540
TIBC, mcg/dL	221.4 (40.1)	246.9 (49.8)	<0.001
UIBC, mcg/dL	153.3 (41.0)	170.9 (56.9)	0.002
Calcium, mg/dL	9.2 (0.7)	9.2 (0.6)	0.531
Phosphate, mg/dL	5.1 (1.5)	5.1 (1.6)	0.924
PTH, pg/mL	336.0 (221.0, 493.0)	324.0 (207.0, 484.0)	0.631
eKt/V	1.4 (0.2)	1.3 (0.2)	0.004
URR	74.0 (5.6)	72.3 (5.5)	0.003
Dialysis vintage, months	35.9 (17.5, 70.3)	44.1 (22.7, 86.4)	0.155

Legend: Values for continuous variables are presented as mean (standard deviation), median (interquartile range), or range. Abbreviations: BMI, body mass index; BP, blood pressure; WBC, white blood cell; RDW, red blood cell distribution width; PTH, parathyroid hormone; TIBC, total iron binding capacity; UIBC, unsaturated iron binding capacity; eKt/V, equilibrated Kt/V; URR, urea reduction ratio

We performed a mediation analysis to see whether EPO usage (with/without) contributed to the increased blood pressure and how much it was through ET-1. We tested this in two models: model 1: included age, sex, race, and BMI; model 2: included age, sex, race, BMI and single pool eKt/V. The average direct effects (ADE) of EPO on blood pressure and the average causal mediation effects of EPO on blood pressure trough ET-1 (ACME) are shown in table 5. Compared with the patients who did not receive EPO treatment, the patients who received EPO had average 7.3 mmHg higher systolic blood pressure (95% CI 1.0-13.5, P=0.016), but EPO showed no effect on diastolic blood pressure after adjustment for age, sex, race, and BMI. The association of ET-1 with higher BP was only mildly mediated by EPO treatment (0.08%, 95%CI 0.002%-0.44%, P=0.040). There was no effect on Diastolic BP. Model 1 and model 2 showed similar results.

Table. 5 The mediation analysis of EPO usage, ET-1 levels, and blood pressure

with *vs.* without EPO therapy	**Model 1 Blood pressure, mmHg**		**Model 2 Blood pressure, mmHg**	
	Systolic BP	**Diastolic BP**	**Systolic BP**	**Diastolic BP**
ADE	7.3 (1.0-13.5, P=0.016)	0.2 (-3.1-3.6, P=0.93)	7.4 (1.4-14.1, P=0.014)	0.5 (-3.0-3.9, P=0.818)
ACME	0.6 (0.02 -1.6, P=0.036)	0.3 (0.008-0.8, P=0.04)	0.6 (-0.03-1.5, P=0.038)	0.3 (-0.01-0.8, P=0.044)
Total Effect	7.9 (1.8-13.9, P=0.004)	0.5 (-2.9-3.9, P=0.76)	8.1 (1.9-14.5, P=0.006)	0.8 (-2.7-4.1, P=0.642)
Proportion of mediated by ET-1	0.08% (0.002%-0.4%, P=0.040)	0.08% (-3.9%-3.0%, P=0.76)	0.07% (0.002%-0.3%, P=0.044)	0.1% (-2.8%-3.5%, P=0.634)

Legend: BP, blood pressure; ADE: average direct effects of EPO on blood pressure; ACME: average causal mediation effects of EPO on blood pressure trough ET-1

Sensitivity analyses were done to check the influence of EPO use on ET-1 and blood pressure. Instead of the one-time point blood pressure close to a blood draw for testing ET-1, average blood pressures four weeks before the blood draw date was used in the analysis. The results were similar.

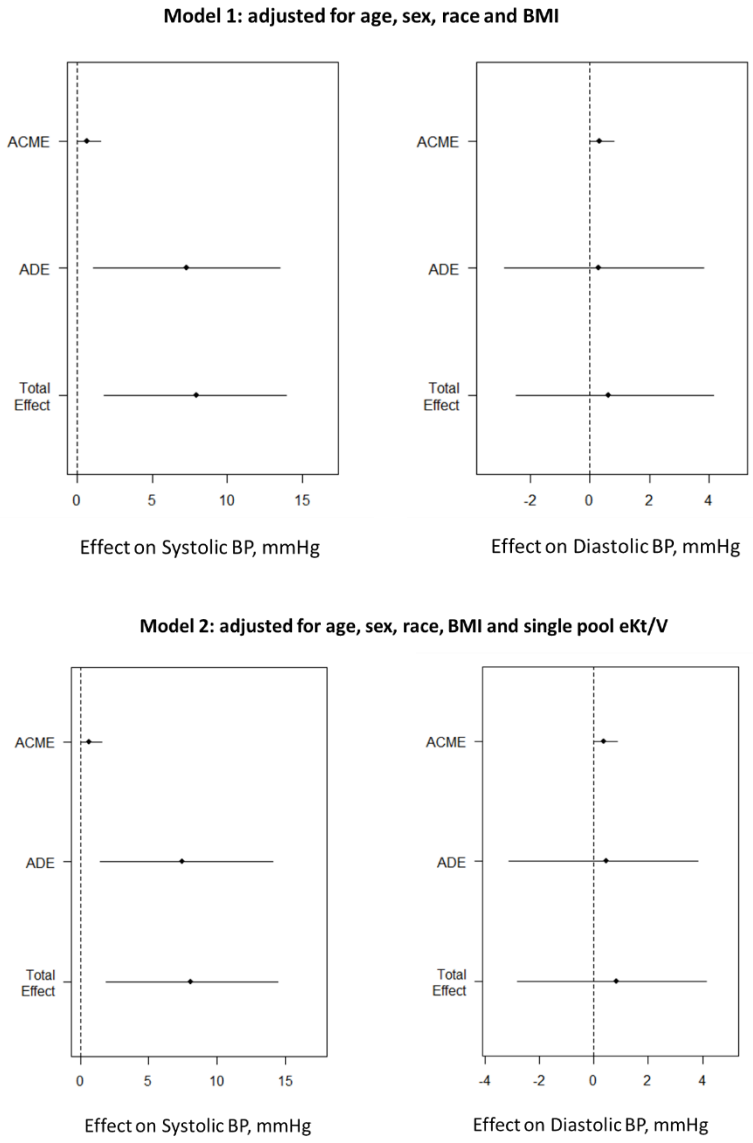
Table. 6. Sensitivity analysis of mediation analysis of EPO usage, ET-1 levels, and blood pressure

with *vs.* without EPO therapy	**Model 1 Average Blood pressure, mmHg**		**Model 2 Average Blood pressure, mmHg**	
	Systolic BP	**Diastolic BP**	**Systolic BP**	**Diastolic BP**
ADE	4.2 (-0.4-8.6, P=0.07)	0.2 (-2.0-2.6, P=0.882)	4.2 (-0.3-8.7, P=0.06)	0.4 (-2.0-2.7, P=0.724)
ACME	0.8 (0.2-1.7, P=0.012)	0.3 (0.04-0.7, P=0.018)	0.8 (0.1-1.6, P=0.024)	0.4 (0.06-0.78, P=0.022)
Total Effect	4.9 (0.3-9.4, P=0.03)	0.6 (-1.6-3.0, P=0.652)	4.9 (0.6-9.4, P=0.032)	0.8 (-1.8-3.1, P=0.532)
Proportion of mediated by ET-1	0.1% (0.01%-0.8%, P=0.042)	0.2% (-5.9%-4.5%, P=0.65)	0.15% (-0.002-0.8%, P=0.052)	0.2% (-3.9%-3.3%, P=0.53)

Legend: ADE: average direct effects of EPO on blood pressure; ACME: average causal mediation effects of EPO on blood pressure trough ET-1

Among the participants who received EPO (n=685), higher dosages correlated with higher plasma ET-1 levels. For every 1-unit increase in natural log-transformed EPO dosage/week, natural log plasma ET-1 levels increased by 0.099 (95% CI 0.064-0.134, P<0.001). That can also be explained as for every one-fold increase in EPO dosage, the ET-1 level increase by 7.1% (95% CI 4.5-9.8, P<0.001). But log-transformed average EPO dosage/week did not show association with Systolic BP or Diastolic BP (P>0.05).

Figure.2. Mediation analysis of EPO usage and ET-1 on blood pressure



Legend: ADE: average direct effects of EPO on blood pressure; ACME: average causal mediation effects of EPO on blood pressure trough ET-1

Discussion

In this study, we found that the plasma ET-1 level was cross-sectional positively correlated with pre-dialysis blood pressure. By the best subset regression which included the fewest variables, diastolic blood pressure, RDW, LDH, and ultrafiltration volume were selected by the model and positively associated with ET-1 level; and negatively associated with hemoglobin, platelet count and iron saturation.

The predominant source of ET-1 in the human body is endothelial cells while a variety of cells can also produce ET-1(61). The kidney is both an important source and target of ET-1. ET-1 probably works as a paracrine/autocrine regulator of renal and intrarenal blood flow, glomerular hemodynamics, and sodium and water homeostasis(62). Elevated ET-1 level has been observed in several types of CKD including diabetic nephropathy, focal segmental glomerulosclerosis, hypertensive nephropathy, sickle nephropathy, and polycystic kidney disease compared to healthy controls(63-69). Regulation of ET-1 mainly takes place as synthesis. ET-1 mRNA is upregulated by transforming growth factor-beta, tumor necrosis factor-alpha, interleukins, insulin, norepinephrine, angiotensin II, and thrombin in many types of cells (1, 70, 71). These factors usually increase in CKD patients while on the other hand, the elevated ET-1 in CKD patients accelerate the progression of kidney disease through reducing renal blood flow, glomerular-filtration rate, renal inflammatory, fibrogenic activity, platelet aggregation, and formation of extracellular matrix protein, etc. (72, 73). Higher levels of ET-1 in HD patients may occur by the same mechanism as in CKD patients, or due to reduced clearance, erythropoiesis stimulating agents (60, 74). Not surprisingly, ET-1 was positively correlated with pre-dialysis blood pressure, consistent with ET-1's regulation of vasoconstriction,

arterial pressure, and water and sodium handling in the kidney (75, 76). The observed association between ET-1 levels and ultrafiltration volume in this study could reflect an increase in ET-1 production in states of volume overload. This is consistent with prior studies showing a positive correlation between endothelial production of ET-1 with mechanical stress(77) and with venous congestion(44).

To our knowledge, our findings of associations between ET-1 levels and hematologic parameters in ESRD patients have not been reported before, and the reasons for such associations have yet to be fully elucidated. In addition to its potent vasoconstrictive function, ET-1 has also been shown to be involved in many inflammatory processes and up-regulation of inflammatory cytokines such as TNF-alpha, IL-1, and IL-6 (78). We observed strong correlations of ET-1 with the severity of anemia, increased platelet counts and iron levels which may reflect the persistent low-grade inflammation in ESRD. These findings suggest a potential connection between ET-1 and cytokine-induced anemia, impaired iron metabolism.

Though it is well-accepted that EPO increases blood pressure and endothelin-1 has been proposed to be one mediator of EPO-induced hypertension, evidence from patients on hemodialysis is lacking. In our cohort, the patients without EPO treatment had higher hemoglobin and hematocrit, which may be attributed to the fact that KIDIGO guidelines recommend that adult patients with hemoglobin less than 10g/dL should get EPO treatment (79). Male patients were less likely to receive EPO, perhaps because men have higher androgen levels which stimulates erythropoiesis (80). In this study, we found that average systolic blood pressure in the EPO treatment group was 7.3 mmHg higher than that

in the no EPO treatment group, but the diastolic blood pressure was similar between the two groups after adjusted for age, sex, race, and BMI. From the baseline characteristics, the patients who did receive EPO treatment showed higher systolic blood pressure, but the diastolic blood pressure was not different between the two groups. The mediation analysis showed the same trend of influence on blood pressure. We did not include hematocrit in the model because hematocrit and blood pressure in our dataset showed the opposite correlation compared with former knowledge. The patients in the EPO treatment group actually showed a lower level of hematocrit, but still had higher blood pressure. EPO therapy will increase of hematocrit which showed to increase the blood viscosity (81). Increase of blood viscosity will cause the compensatory of blood pressure elevating. For this case, the patients without EPO patients had a higher hematocrit, and their blood viscosity which was not measured in our study should be higher than that in the patients with EPO treatment.

The mechanisms of EPO-induced hypertension are not well understood. Several molecules including nitric oxide, endothelin, prostanoids, and renin-angiotensin have been shown to be involved in the pathogenesis of EPO-induced hypertension from preclinical studies (82-86). EPO also can directly constrict the vascular smooth muscle cells of arteries without endothelium (87). EPO-treated rats with reduced renal mass had ET-1 increase in the aortic content (88). Whether ET-1 plays a mediating role in EPO-induced hypertension from human research remains controversial. One smaller study of dialysis patients who started receiving darbepoetin showed an increase of ET-1 and increased systolic and diastolic blood pressures similarly (60). However, other studies found no increase in ET-1 after EPO treatment (89, 90). In our analysis, EPO treatment groups had a higher SBP and

higher levels of ET-1, but ET-1 only contributed 0.08% of the average systolic blood pressure increase in the EPO treatment group. Since several other pathways are implicated in EPO-induced hypertension, such as renin, endothelin/nitric oxide ratio, asymmetric dimethylarginine, further studies should explore the role of these alternate pathways in EPO-induced hypertension. The dosage of EPO was not associated with systolic blood pressure or diastolic blood pressure but was associated with elevated ET-1 levels in our study. The results were similar to the trials. Though the blood pressure was not the primary outcome, some EPO/high target versus EPO low target randomized trials had a description of blood pressure. The blood pressures were not different between the high EPO dose and low EPO dose groups (91-94).

Our study has several limitations. First, the ET-1 level was only tested at a one-time point before dialysis, and we do not know about the variability of ET-1 within patients. Second, since this is a cross-sectional study, we cannot draw a causal association between the characteristics and ET-1 levels. Third, since the accurate time initiation of EPO treatment is now available in the dataset which means that the blood pressure was not available either, we cannot perform an inverse probability of treatment weighting (IPW) to calculate the average difference of blood pressure between the patients who received EPO treatment and the patients who did not receive EPO treatment. Overall, for the first time, our research provides risk factors for elevated ET-1 and finds a mild mediation effect of ET-1 in EPO-induced hypertension.

Discussion and perspectives

End-stage renal disease (ESRD) is a worldwide public health problem. More than 2 million people worldwide are being treated for ESRD. The main treatment for ESRD is still hemodialysis (HD)(95). Although some progress has been made in the past decades, HD patients continue to experience significant mortality and morbidity. According to the USRDS annual data report, mortality rates were still high as 136 per 1,000 patient-years for HD patients (96). The risk prediction and control for the mortality in HD patient did not meet the demand.

The kidney is producing abundant ET-1, and almost every type of cells in the kidney expresses ET-1 receptor (75). ET-1 has shown to be associated with kidney disease progression including glomerulosclerosis, interstitial fibrosis both from preclinical and clinical studies (23, 68, 97). Based on the cumulative evidence, clinical trials which target ET-1 receptor have been launched among CKD patients, and the results were promising (98). ET-1 increases strikingly among hemodialysis patients. To our knowledge, this is the first research that illustrates that higher ET-1 levels associated with adverse events in hemodialysis patients independent of previously described risk factors. Also, we observed strong correlations of ET-1 with the severity of anemia, increased platelet counts and iron levels which may reflect the persistent low-grade inflammation in ESRD suggesting a potential connection between ET-1 and cytokine-induced anemia, impaired iron metabolism. Although our findings cannot establish causality between ET-1 and adverse outcomes in ESRD, our findings raise the question of whether ET-1 antagonism could be a promising therapeutic approach in treating ESRD patients. If our findings are confirmed in

other studies, randomized controlled trials should be conducted to test whether ET-1 antagonism may be a promising pharmacological intervention in ESRD.

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