Published in final edited form as:

Int J Cardiol. 2014 September ; 176(1): 99–109. doi:10.1016/j.ijcard.2014.06.062.

Increased Concentration of Circulating Angiogenesis and Nitric Oxide Inhibitors Induces Endothelial to Mesenchymal Transition and Myocardial Fibrosis in Patients with Chronic Kidney Disease

David M. Charytan, MD, MSc¹, Robert Padera, MD, PhD², Alexander M. Helfand, BA¹, Michael Zeisberg, MD³, Xingbo Xu, PhD⁴, Xiaopeng Liu, MSc⁴, Jonathan Himmelfarb, MD⁵, Angeles Cinelli, BA¹, Raghu Kalluri, MD, PhD⁶, and Elisabeth M. Zeisberg, MD^{4,7}

¹Renal Division, Department of Medicine, Brigham & Women's Hospital, Boston, MA

²Department of Pathology, Brigham & Women's Hospital, Boston, MA

³Departments of Nephrology, Rheumatology, University Medical Center, Georg-August University, Göttingen, Germany

⁴Departments of Cardiology and Pneumology University Medical Center, Georg-August University, Göttingen, Germany

⁵Department of Medicine, University of Washington School of Medicine

⁶Department of Cancer Biology, MD Anderson Cancer Center, Houston, Texas

⁷DZHK (German Centre for Cardiovascular Research), partner site Göttingen, Germany

Abstract

Background—Sudden cardiovascular death is increased in chronic kidney disease (CKD). Experimental CKD models suggest that angiogenesis and nitric oxide (NO) inhibitors induce myocardial fibrosis and microvascular dropout thereby facilitating arrhythmogenesis. We undertook this study to characterize associations of CKD with human myocardial pathology, NO-related circulating angiogenesis inhibitors, and endothelial cell behavior.

Methods—We compared heart (n=54) and serum (n=162) samples from individuals with and without CKD, and assessed effects of serum on human coronary artery endothelial cells (HCAEC) *in vitro*. Left ventricular fibrosis and capillary density were quantified in post-mortem samples. Endothelial to mesenchymal transition (EndMT) was assessed by immunostaining of post-mortem samples and RNA expression in heart tissue obtained during cardiac surgery. Circulating

© 2014 Elsevier Ireland Ltd. All rights reserved.

Corresponding Author: David Charytan, MD MSc, Renal Division, Brigham & Women's Hospital, 1620 Tremont Street, 3rd Floor, Boston, MA 02120, Phone: (617) 525-7718, Fax: (617) 525-7752, dcharytan@partners.org.

Disclosures: None declared. Author contributions-DMC and EMZ-study design, conduct of experiments, and analysis of data, drafting of manuscript. RP, RK, AMH-study design, conduct of experiments, and analysis of study. MZ and JH-analysis of data and drafting of manuscripts. AC, XX, XL-conduct of experiments and analysis of data. All authors approved the final draft.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

asymmetric dimethylarginine (ADMA), endostatin (END), angiopoietin-2 (ANG), and thrombospondin-2 (TSP) were measured, and the effect of these factors and of subject serum on proliferation, apoptosis, and EndMT of HCAEC were analyzed.

Results—Cardiac fibrosis increased 12% and 77% in stage 3-4 CKD and ESRD and microvascular density decreased 12% and 16% vs. preserved renal function. EndMT-derived fibroblast proportion was 17% higher in stage 3-4 CKD and ESRD ($P_{\text{trend}}=0.02$). ADMA, ANG, TSP, and END concentrations increased in CKD. Both individual factors and CKD serum increased HCAEC apoptosis ($P=0.02$), decreased proliferation ($P=0.03$), and induced EndMT.

Conclusions—CKD is associated with an increase in circulating angiogenesis and NO inhibitors, which impact proliferation and apoptosis of cardiac endothelial cells and promote EndMT, leading to cardiac fibrosis and capillary rarefaction. These processes may play key roles in CKD-associated CV disease.

Keywords

CKD; ESRD; fibrosis; endothelial to mesenchymal transition; cardiovascular disease; angiogenesis inhibitor

1. Introduction

Individuals with chronic kidney disease (CKD) have high risks of developing and dying from cardiovascular (CV) disease and these risks are not fully explained by traditional risk factors[1]. Strong associations of novel factors with CV events, a failure of standard therapies to substantively impact mortality in advanced CKD, and an outsize risk of sudden death relative to myocardial infarction suggest that unique features underlie CVD in the setting of uremia[2].

Experimental models of uremia are characterized by myocardial fibrosis, loss of myocardial capillaries, and inhibition of ischemia driven neo-angiogenesis[3, 4]. These changes increase capillary to myocyte distance, alter oxygen delivery, and disrupt myocardial conduction, thereby facilitating propagation of arrhythmias. Experimental studies also demonstrate altered nitric oxide (NO) bioavailability in uremia[5] which may induce secondary changes in the activity and concentration of additional angiogenesis inhibitors[6-8] thereby contributing to the observed myocardial fibrosis and capillary rarefaction. However, similar data in human disease remains sparse. We undertook this study to characterize for the first time changes in human myocardial pathology across the spectrum of CKD and assess associations with NO-related circulating angiogenesis inhibitors and their effects on human coronary artery endothelial cells (HCAEC).

2. Materials and Methods

2.1 Autopsy Cohort

Autopsies performed at Brigham & Women's Hospital (BWH) between 2004-2006 (n=45) were included. Cases without sufficient data to estimate kidney function, history of acute kidney injury lasting >1 week, cardiac transplant, active cancer, prior thoracic irradiation, treatment with anthracyclines, congenital heart disease, idiopathic or viral cardiomyopathy,

or insufficient tissue were excluded. Medical history and laboratory data were extracted from clinical records. Kidney function was estimated from outpatient serum creatinine or from the lowest stable value (replicated on 2 occasions) if outpatient values within 4 months were unavailable.

2.2 Serologic Cohort

Individuals 18-80 years old were recruited from the coronary angiography and outpatient nephrology clinics at BWH (n=162). Individuals with acute kidney injury, history of thoracic radiation, malignancy, receiving anti-angiogenic or immunosuppressive therapy, or requiring urgent angiography were excluded. Angiography subjects were excluded if they had a history of coronary bypass surgery. Serum and plasma were collected prior to angiography, centrifuged within 15 minutes at 1000g, and stored at -80°C. Clinical data were obtained through interview and chart review. Serum creatinine, albumin, and hemoglobin were measured on the day of enrollment in the clinical laboratory. Cholesterol and urinary albumin and creatinine measurements within 6 months prior to enrollment were also recorded but urine was not collected.

In both cohorts, estimated glomerular filtration rate (eGFR) was calculated using the modified MDRD equation[9]. Given the sample size, individuals with preserved renal function and eGFR ≥ 90 (prospective cohort) or ≥ 60 mL/min/1.73m² (autopsy) were considered jointly as preserved function/stage 1 or 1-2 CKD, according to standard definitions[10]. Stage 3-4 CKD was similarly combined. Patients with dialysis dependent ESRD were assigned a creatinine of 8.0 mg/dL (707.2 μ mol/L) and eGFR of 2 mL/min/1.73m².

2.3 Cardiac Surgery Cohort

Right atrial appendage was harvested as discarded tissue from 9 individuals undergoing non-emergent cardiac surgery. Appendages were snap frozen and stored at -80°C. Renal function was estimated using preoperative measurements of serum creatinine and clinical history was obtained via interview and chart review.

2.4 Circulating Factors

Samples were batch analyzed in duplicate for serum endostatin (END) and angiopoietin-2 (ANG) using Quantikine[®] ELISA (R&D Systems) with intra- and inter-assay coefficient of variations (CV) <7.0% and 10.5%. Thrombospondin-2 (TSP) was measured using Luminex[®] assays (R&D Systems) with CVs <8.7% and 16.4%, respectively. Plasma asymmetric dimethyl arginine (ADMA) was measured by HPLC as described by Teerlink[11] (intra and inter-assay CV <3.5%).

2.5 Microscopic Analyses

Microscopic analyses were blinded. Five μ m paraffin-embedded left ventricular (LV) sections were stained with hematoxylin or Masson's trichrome. After microscopic review to exclude infarcted myocardium, sections were incubated with phosphate buffered saline (negative control) or anti-CD31 antibodies (Pharmingen), followed by anti-rat or Envision[™] anti-rabbit secondary antibodies (Dako), and developed with DAB (Dako).

An Olympus BX41 microscope and camera were used to capture digital images. Image Pro 6.2 (Media Cybernetics) was used to digitally label blue pixels and measure collagen deposition as the percentage of the total myocardium within 10 representative fields (100x magnification). Microvascular density was measured at 200x (10 fields/case). Myocytes and CD-31 positive, tubular structures of appropriate diameter were manually counted using digital calipers. Pixels enclosed within each cell were labeled, and the area occupied was measured.

Endothelial to mesenchymal transition (EndMT) was assessed in 5 μ m paraffin sections by double labeling with endothelial (CD31) and fibroblast (FSP1) markers. Following antigen retrieval in proteinase K (Dako) at 37°C, sections were incubated with anti-CD31 (Dako) and anti-FSP1 (Dako) and fluorescent-conjugated secondary antibodies (Invitrogen) and counterstained with DAPI (Invitrogen and/or Vector Labs). Sections were examined at 630x using confocal microscopy (Zeiss).

2.6 RNA extraction and Quantitative PCR (qPCR) for EndMT

Total RNA was extracted using Trizol Reagent (Life technologies). RNA purification was performed using a PureLink RNA Mini Kit (Ambion) following the manufacturers' protocol. 250ng of total RNA was digested with DNaseI (Sigma) and used for cDNA synthesis using SuperScript II Reverse Transcriptase (Life Technologies) according to manufacturers' instructions. For qPCR analysis, diluted cDNA (1/10) was used as a template in a Fast SYBR Green Master Mix (Life Technologies) and run in StepOne Plus Instrument (Life Technologies). Primers were designed and purchased from PrimerDesign. TWIST, SLUG, and SNAIL RNA expression were measured as indices of EndMT[12]. All qPCR data for RNA expression analysis (two or more biological replicates with three technical replicates each) were calculated using the delta delta Ct method and standardized to the reference gene GAPDH expression.

2.7 Proliferation, Apoptosis, and EndMT Assays

HCAEC (Lonza), passage 5, were seeded at 37°C in 5% CO₂ on 96-well polystyrene plates (Becton Dickinson, Falcon) at 8×10^4 cells/mL, in EBM-2 medium (Lonza) supplemented with 2% fetal bovine serum (FBS, Sigma). After 24 hours, medium was replaced and cells were cultured in duplicate for 24 hours in 2% subject serum or in triplicate with 2% FBS spiked with physiologic concentrations of ADMA (Sigma), recombinant human END (Prospec Bio, Sigma), ANG, or TSP (both from R&D Systems). A proliferation detection agent, WST-1 (Roche), was added after 20 hours and cells were cultured an additional 4 hours before measurement on a Versamax™ microplate reader (Molecular Devices) at 440 nm.

Apoptosis was measured in triplicate on HCAEC passage 5-7 on opaque polystyrene plates (Costar) using 5% FBS. Medium was replaced after 24 hours with 5% subject serum or 5% FBS spiked with ADMA or recombinant factors. Human TNF-alpha (0.5-20 ng/mL, Promega) was used as a positive control. After 21 hours, Caspase-Glo 3/7 Reagent (Promega) was added and plates were incubated at room temperature for 3 hours. Luminescence was measured with a Spectramax-M5 microplate reader (Molecular Devices).

EndMT was measured in triplicate on HCAEC passage 5-7. As above, medium was replaced after 24 hours and spiked with ADMA or recombinant factors. Cells were harvested after 48 hours and total RNA was analyzed as above.

2.8 Statistical Analysis

Data are presented as mean $\pm$ standard deviation (SD), n (%) or median (inter-quartile range, IQR). Baseline differences were compared using ANOVA and non-parametric trend tests for continuous data and Chi-square tests for count data. Outcome variables including END, TSP, ANG and ADMA concentrations were logarithmically transformed to preserve normality. Differences across categories of CKD were assessed with ANOVA and the post-hoc Tukey-Kramer test to assess pairwise differences and non-parametric trend tests or orthogonal polynomial regression to assess trends. Continuous correlations were measured with the Pearson coefficient. Multivariable linear regression models were constructed to adjust for confounding factors. Model fit was tested graphically, by inspecting residuals, and by testing model specification. Analyses were performed in STATA 9.0 and 13.0 (STATA Corp.) with $P < 0.05$ considered significant.

3. Results

3.1 Evaluation of Cardiac Fibrosis

The risk of sudden cardiac death increases dramatically as GFR declines[13]. Sudden cardiac death and overall mortality on the other hand are strongly associated with the presence of cardiac fibrosis [14]. We therefore aimed to systematically evaluate amount of cardiac fibrosis over various stages of CKD by analyzing heart tissue obtained from autopsy.

Among our autopsy cohort of 45 individuals, we identified 21 subjects with preserved kidney function, 17 with stage 3-4 CKD, and 7 with ESRD (Table 1). Mean eGFR was 101.7 ± 34.2 in the group with preserved kidney function and 37.6 ± 16.2 ml/min/1.73m² in those with stage 3-4 CKD. Individuals with ESRD (66.6 ± 5.6 years) and stage 3-4 CKD (72.8 ± 10.5) were older than individuals with preserved function (58.0 ± 15.2 , $P = 0.003$). Diabetes ($P < 0.001$) and hyperlipidemia ($P = 0.03$) were more frequent, and there were nonsignificant trends towards a higher frequency of coronary disease ($P = 0.13$) and prior MI ($P = 0.12$) as CKD progressed.

The extent of interstitial fibrosis increased by 12% and 77% in individuals with stage 3-4 CKD and ESRD, respectively ($P_{\text{trend}} = 0.003$, Table 2 and Figure 1 A, C and G). Microvascular supply decreased by 12% and 16% in stage 3-4 CKD and ESRD, respectively ($P_{\text{trend}} = 0.04$, Figure 1D-F, H). Associations between CKD group and percent fibrosis ($P = 0.04$) or microvascular supply ($P = 0.03$) remained significant in models adjusting for age, sex, diabetes, and hypertension, and in models adjusting for anemia or the use of either angiotensin converting enzyme inhibitors or angiotensin receptor blockers (Supplementary Tables A.2 and A.3). Numerical decreases in cardiomyocyte density were not significant (Figure 1 I,J). The number of fibroblasts (detected by FSP-1 positivity on confocal microscopy, Figure 2) increased with CKD severity and was 57% higher with ESRD than with preserved renal function ($P_{\text{trend}} = 0.04$, Figure 2 A, B). Given the concurrent fibrosis and microvascular rarefaction, we analyzed the fibroblast-population derived from endothelial to

mesenchymal transition (as evidenced by dual-positivity for FSP-1 and CD-31), as this mechanism potentially accounts for both findings. The proportion of CD31/FSP1 double positive fibroblasts likely derived from EndMT was 17% higher in stage 3-4 CKD and ESRD compared to preserved renal function ($P_{\text{trend}}=0.01$). Continuous measures of eGFR (mL/min/1.73m^2) were correlated with fibrosis, the number of microvessels/cross-sectional field, myocyte density, and the ratio of double-positive to all FSP-1⁺ cells/field (Supplementary Table A.1).

In order to further investigate the confocal microscopy findings from the post-mortem samples suggesting an increase in myocardial EndMT with progressive renal impairment we performed expression analysis of EndMT marker genes using snap frozen atrial appendage tissue harvested from 9 individuals—3 with preserved renal function, 3 with CKD, and 3 with ESRD—undergoing cardiac surgery. Despite the small sample size, the results were suggestive of an increase in SLUG, TWIST and SNAIL expression consistent with an increase in myocardial EndMT with worsening renal function (Figure 2E-G).

3.2 Effects of Uremic Serum on Human Coronary Endothelial Cells

We aimed to test whether uremic serum could mediate such EndMT. Among 162 individuals enrolled in our serologic cohort, 30 had preserved renal function, 58 stage 2, 60 stage 3-4, and 14 stage 5 CKD, the majority of whom were on dialysis (Table 3). Compared to the individuals with preserved function (57.2 ± 8.3 years old), age was higher in subjects with stage 2 (61.1 ± 11.4 years) and with stage 3-4 CKD (67.0 ± 11.8 years) but was decreased among the individuals with stage 5 CKD (48.9 ± 20.0 years, $P<0.001$). Diabetes ($P=0.03$) and hypertension ($P=0.03$) were more frequent with more advanced CKD (Table 3).

We first assessed if serum from patients with CKD affected human coronary endothelial cells differently than serum from healthy individuals. Upon treatment with CKD serum, cell number decreased and HCAECs changed towards more spindle-shaped cell morphology as compared to treatment with serum from healthy individuals (Figure 3A). Accordingly, quantification of apoptosis showed an increase ($P_{\text{trend}}=0.02$) while proliferation of HCAEC decreased ($P_{\text{trend}}=0.03$) after incubation in serum from individuals with more severe CKD (Table 4, Figure 3 B-C). In addition, exposure to serum from individuals with more severe CKD also increased expression of the EndMT-related transcripts TWIST ($P_{\text{trend}}=0.03$) and SNAIL in HCAEC ($P_{\text{trend}}=0.012$, Figure 3D).

3.3 Serologic Factors affecting Human Coronary Endothelial Cells in CKD

In CKD the overall production of NO is decreased [5]. Several serological factors have been found to be associated with both lower NO and increased cardiovascular disease in animal models, such as the NO-synthase inhibitor ADMA and the angiogenesis inhibitors ANG, TSP and END [6-8, 15-23]. In order to identify specific serologic factors which could mediate the observed effect of uremic serum on coronary endothelial cells, we measured serum ADMA, ANG, TSP and END in our cohort ($n=162$). All were increased in serum of patients with CKD: END concentration was nearly 10-fold higher in stage 5 CKD (879.6 ng/mL , IQR $631.4-1129.3$) than with preserved renal function (91.1 ng/mL , IQR $69.4-109.6$)

and concentrations of END, TSP, ANG and ADMA increased significantly across categories of CKD and were independently associated with renal function (Table 4, Figure 4, Supplementary Table A.3). END, ANG and ADMA concentrations were significantly higher in stage 5 CKD than with preserved function. Similarly, END, TSP and ANG concentrations were higher with stage 5 CKD compared with preserved kidney function. ADMA concentration was correlated with END ($r=0.34$, $P<0.001$) and ANG ($r=0.38$, $P<0.001$).

HCAEC apoptosis and END, TSP, ANG, and ADMA concentrations were independently associated with CKD class in multivariable models adjusted for age, race, sex, diabetes, hypertension, smoking, history of myocardial infarction, recruitment site, and presence of acute coronary syndrome. However, the association with HCAEC proliferation was attenuated after multivariable adjustment (Supplementary Table A.2). Results were similar with further adjustment for anemia or use of angiotensin blockers and angiotensin converting enzyme inhibitors, but the association of CKD class with TSP concentration was slightly attenuated.

To assess whether these factors could mediate the effects of uremic serum on apoptosis, proliferation, and EndMT of HCAEC, we tested the effects of a wide range of concentrations (which included the previously documented range among healthy and CKD patients) of purified, recombinant ADMA, END, TSP and ANG on HCAEC. Recombinant ANG increased apoptosis of HCAEC at all concentrations tested (Figure 5B) and both ANG ($P_{\text{trend}}<0.001$) and TSP ($P_{\text{trend}}<0.001$) increased apoptosis at increasing concentrations within the physiologic range (Figure 5B,E). Similarly, HCAEC proliferation was inversely proportional to the concentrations of ADMA ($P_{\text{trend}}<0.001$), ANG ($P_{\text{trend}}=0.05$) and TSP ($P_{\text{trend}}=0.002$) added to the medium (Figure 5A,D,G). Each of these proteins also induced EndMT of HCAEC, as reflected by increased expression of TWIST and SLUG RNA (Figure 5C,F,I,L). In contrast, physiologic concentrations of END decreased apoptosis and increased HCAEC proliferation ($P<0.001$, Figure 5J,K).

4. Discussion

We found that myocardial fibrosis and EndMT increased while microvascular supply decreased significantly with CKD severity. In addition, the concentration of circulating angiogenesis and NO inhibitors increased with CKD severity while serum from patients with more severe CKD inhibited proliferation and increased apoptosis of cultured coronary endothelial cells. Finally, ADMA, ANG, TSP and END had qualitatively similar effects on cultured endothelial cells as uremic serum.

CKD is strongly associated with CVD, especially heart failure and sudden death[1]. Experimental studies suggest that renal impairment inhibits ischemia-driven angiogenesis[4] and induces myocardial capillary rarefaction and fibrosis[3], but evidence of these processes in humans is limited. In one study comparing 9 dialysis patients with 9 hypertensive and 10 non-hypertensive controls—all free from coronary disease and with non-CV causes of death—LV capillary density decreased by 49% and 21% and interstitial tissue increased by 65% and 44% compared with normal and hypertensive controls, respectively[24]. Conversely, in endomyocardial biopsies of 90 patients with dilated cardiomyopathy, myocyte diameter was

significantly increased in the dialysis group. However, in this cohort with advanced cardiomyopathy, LV fibrosis did not differ between dialysis patients and controls[25].

Our findings add to these studies by demonstrating significant increases in myocardial fibrosis and capillary rarefaction in a less highly-selected population of dialysis patients, and by showing that changes in capillary supply and myocardial fibrosis begin relatively early in CKD before accelerating in ESRD. Whether differences in technique (measurement of myocyte area vs. diameter), patient population, or statistical power explain the divergent findings on myocyte size requires further study. Finally, our findings of an increase in cells dual positive for endothelial and fibroblastic markers (as well as trends consistent with an increase in SNAIL and SLUG mRNA expression) provide the first evidence that EndMT [26] with transformation of endothelial cells into fibroblasts has a role in the capillary rarefaction and myocardial fibrosis characterizing the uremic myocardium.

NO homeostasis is abnormally regulated in experimental uremia[5], and in experimental models NO deficiency induces myocardial fibrosis and capillary rarefaction[27] while lowering NO concentrations induces END, ANG, and TSP synthesis and the exocytosis of ANG from endothelial cells[6-8, 15]. Our observation that ADMA—a potent inhibitor of NO synthase—increases with CKD severity and is accompanied by analogous changes in END, ANG, and TSP, suggests a model of CVD in CKD in which NO deficiency—partly driven by increased ADMA—leads to myocardial fibrosis and microvascular dropout through direct effects on endothelial cells and fibroblasts and by indirectly stimulating production of additional, potent angiogenesis inhibitors (Figure 8). These novel pathways are likely to synergize with other traditional risk factors common in CKD such as volume overload, anemia, as well as abnormalities in insulin signaling, parathyroid hormone, calcium and phosphorous.

These results are consistent with prior studies demonstrating that ADMA and ANG-2 concentrations are increased in CKD and associated with higher risks of CV and all-cause death[16-19]. They also confirm studies demonstrating that END concentration rises in both pre-dialysis and dialysis-dependent CKD[20, 21]. Our study extends these observations by demonstrating the independence of these associations from other standard risk factors and by showing analogous changes in an additional potent angiogenesis inhibitor, TSP, which is known to promote renal capillary rarefaction and to promote fibrosis and inhibit vascularization of experimental cardiac allografts[22, 23], but whose association with kidney function has not been previously assessed[20, 21] [22, 23].

We also demonstrated potent effects of uremic serum on endothelial cells as well as specific effects of ADMA, ANG, END, and TSP on endothelial apoptosis, proliferation, and EndMT at their circulating concentrations. The *in vitro* and *in vivo* evidence of EndMT that we observed suggest that induction of EndMT with a resultant loss of endothelial cells and increase in myocardial fibroblasts (together with endothelial apoptosis and inhibited proliferation) play key roles in the characteristic myocardial pathology of uremia and are consistent with experimental studies suggesting a mechanistic role for EndMT in cardiac fibrosis[26].

In contrast to prior studies[28, 29], END did not impair proliferation or promote apoptosis at the studied concentrations. This could reflect a difference in cell lines (HCAEC vs. cow pulmonary artery and human umbilical vein cells), but our preliminary observations showed inhibition of HCAEC proliferation for concentrations of END above 1 μ g/mL (data not shown) suggesting that the lower concentrations tested (ng/mL vs. μ g/mL) underlie the divergence. Our data thus suggests a complex biology in which increasing END levels initially increase proliferation and inhibit apoptosis, but which is reversed at higher concentrations. Although the moderate rise in *circulating* END in CKD is thus unlikely to be a major factor underlying endothelial apoptosis or proliferation, these levels may induce EndMT. Furthermore, local myocardial concentrations of END may also be crucial. Tissue levels of endostatin are inversely correlated with myocardial capillary density in experimental models[30] while inhibition of NO synthesis increases both *in vitro* production of END by endothelial cells and *in vivo* tissue END levels[6]. Thus, high circulating levels of ADMA in CKD likely increase both serum and *tissue* END concentration. Myocardial END concentrations derived from local endothelial synthesis may thus be sufficient to promote apoptosis and inhibit endothelial proliferation regardless of circulating concentrations. Further studies to fully elucidate END's role and to measure myocardial tissue levels in CKD are warranted.

4.1 Strengths and Limitations

This study has several strengths. In particular we used a multifaceted approach with consistent data from histopathological (autopsy), serological, and *in vitro* studies, encompassing cell biology and cardiac structure and function. There were several limitations: The numbers of ESRD patients was small, and additional studies including more dialysis patients are needed. The number of post-mortem samples was particularly small. Our ability to simultaneously adjust for relevant covariates in the analysis of these samples was limited, and although our findings appeared robust, we cannot rule out the possibility that residual confounding may partly explain the observed associations with CKD. Our findings should thus be confirmed using detailed, multi-variable adjustment and larger multi-center cohorts before they can be broadly generalized. Finally, our studies were associative in nature. Although interventional studies are needed for causality, our studies provide novel insights into the potential mechanisms underlying uremic CVD.

4.2 Conclusions

In conclusion, we found significant associations between the severity of CKD, myocardial fibrosis and capillary rarefaction as well as significant increases in the circulating concentrations of ADMA, END, TSP and ANG in individuals with CKD. Our human data support experimental animal studies suggesting that capillary rarefaction and fibrosis underlie the high risk of CV death in CKD, particularly in ESRD, while suggesting an important role for ADMA-related inhibition of NO homeostasis and related increases in END, TSP, ANG and EndMT in those pathologic changes (Figure 6). Further studies are needed to determine whether restoring NO homeostasis or inhibiting ADMA, END, TSP, or ANG can improve CV histology or outcomes in CKD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding: This work was supported by funds of the University Medical Center of Göttingen (EMZ and MZ); the Paul Teschan Research Fund; the Carl Gottschalk Award of the American Society of Nephrology; the American Heart Association Scientist Development Grant [0735638N] (DMC), NIH RO1 HL070938 (JH), grants ZE523/3-1 and ZE523/2-1 by the Deutsche Forschungsgemeinschaft (MZ), the Cancer Prevention and Research Institute of Texas, the Metastasis Research Center at MD Anderson Cancer Center, and NIH grants DK055001, DK081576, CA125550, CA155370, CA151925 and CA163191 (RK). Elisabeth Zeisberg is further supported by the SFB1002/C01 DFG grant.

References

1. Go AS, Chertow GM, Fan D, McCulloch CE, Hsu CY. Chronic kidney disease and the risks of death, cardiovascular events, and hospitalization. *N Engl J Med*. 2004; 351:1296–305. [PubMed: 15385656]
2. Herzog CA, Asinger RW, Berger AK, Charytan DM, Diez J, Hart RG, et al. Cardiovascular disease in chronic kidney disease. A clinical update from Kidney Disease: Improving Global Outcomes (KDIGO). *Kidney Int*. 2011; 80:572–86. [PubMed: 21750584]
3. Amann K, Neimeier KA, Schwarz U, Tornig J, Matthias S, Orth SR, et al. Rats with moderate renal failure show capillary deficit in heart but not skeletal muscle. *Am J Kidney Dis*. 1997; 30:382–8. [PubMed: 9292567]
4. Jacobi J, Porst M, Cordasic N, Namer B, Schmieder RE, Eckardt KU, et al. Subtotal nephrectomy impairs ischemia-induced angiogenesis and hindlimb re-perfusion in rats. *Kidney Int*. 2006; 69:2013–21. [PubMed: 16641920]
5. Baylis C. Nitric oxide deficiency in chronic kidney disease. *Am J Physiol Renal Physiol*. 2008; 294:F1–9. [PubMed: 17928410]
6. O'Riordan E, Mendelev N, Patschan S, Patschan D, Eskander J, Cohen-Gould L, et al. Chronic NOS inhibition actuates endothelial-mesenchymal transformation. *Am J Physiol Heart Circ Physiol*. 2007; 292:H285–94. [PubMed: 16963618]
7. Bhandari V, Choo-Wing R, Harijith A, Sun H, Syed MA, Homer RJ, et al. Increased hyperoxia-induced lung injury in nitric oxide synthase 2 null mice is mediated via angiotensin II. *Am J Respir Cell Mol Biol*. 2012; 46:668–76. [PubMed: 22227562]
8. MacLauchlan S, Yu J, Parrish M, Asoulin TA, Schleicher M, Krady MM, et al. Endothelial nitric oxide synthase controls the expression of the angiogenesis inhibitor thrombospondin 2. *Proc Natl Acad Sci U S A*. 2011; 108:E1137–45. [PubMed: 21949402]
9. Levey AS, Coresh J, Greene T, Stevens LA, Zhang YL, Hendriksen S, et al. Using standardized serum creatinine values in the modification of diet in renal disease study equation for estimating glomerular filtration rate. *Ann Intern Med*. 2006; 145:247–54. [PubMed: 16908915]
10. Eknoyan G, Hostetter T, Bakris GL, Hebert L, Levey AS, Parving HH, et al. Proteinuria and other markers of chronic kidney disease: a position statement of the national kidney foundation (NKF) and the national institute of diabetes and digestive and kidney diseases (NIDDK). *Am J Kidney Dis*. 2003; 42:617–22. [PubMed: 14520612]
11. Teerlink T. HPLC analysis of ADMA and other methylated L-arginine analogs in biological fluids. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2007; 851:21–9.
12. Medici D, Kalluri R. Endothelial-mesenchymal transition and its contribution to the emergence of stem cell phenotype. *Semin Cancer Biol*. 2012; 22:379–84. [PubMed: 22554794]
13. Pun PH, Smarz TR, Honeycutt EF, Shaw LK, Al-Khatib SM, Middleton JP. Chronic kidney disease is associated with increased risk of sudden cardiac death among patients with coronary artery disease. *Kidney Int*. 2009; 76:652–8. [PubMed: 19536082]

14. Gulati A, Jabbour A, Ismail TF, Guha K, Khwaja J, Raza S, et al. Association of fibrosis with mortality and sudden cardiac death in patients with nonischemic dilated cardiomyopathy. *JAMA*. 2013; 309:896–908. [PubMed: 23462786]
15. Matsushita K, Morrell CN, Cambien B, Yang SX, Yamakuchi M, Bao C, et al. Nitric oxide regulates exocytosis by S-nitrosylation of N-ethylmaleimide-sensitive factor. *Cell*. 2003; 115:139–50. [PubMed: 14567912]
16. Fleck C, Janz A, Schweitzer F, Karge E, Schwertfeger M, Stein G. Serum concentrations of asymmetric (ADMA) and symmetric (SDMA) dimethylarginine in renal failure patients. *Kidney Int Suppl*. 2001; 78:S14–8. [PubMed: 11168976]
17. Zoccali C, Bode-Boger S, Mallamaci F, Benedetto F, Tripepi G, Malatino L, et al. Plasma concentration of asymmetrical dimethylarginine and mortality in patients with end-stage renal disease: a prospective study. *Lancet*. 2001; 358:2113–7. [PubMed: 11784625]
18. David S, Kumpers P, Lukasz A, Fliser D, Martens-Lobenhoffer J, Bode-Boger SM, et al. Circulating angiopoietin-2 levels increase with progress of chronic kidney disease. *Nephrol Dial Transplant*. 2010; 25:2571–6. [PubMed: 20179005]
19. David S, John SG, Jefferies HJ, Sigrist MK, Kumpers P, Kielstein JT, et al. Angiopoietin-2 levels predict mortality in CKD patients. *Nephrol Dial Transplant*. 2012; 27:1867–72. [PubMed: 21976741]
20. Chen J, Hamm LL, Kleinpeter MA, Husserl F, Khan IE, Chen CS, et al. Elevated plasma levels of endostatin are associated with chronic kidney disease. *Am J Nephrol*. 2012; 35:335–40. [PubMed: 22456114]
21. Watorek E, Paprocka M, Dus D, Kopec W, Klinger M. Endostatin and vascular endothelial growth factor: potential regulators of endothelial progenitor cell number in chronic kidney disease. *Pol Arch Med Wewn*. 2011; 121:296–301. [PubMed: 21909059]
22. Daniel C, Amann K, Hohenstein B, Bornstein P, Hugo C. Thrombospondin 2 functions as an endogenous regulator of angiogenesis and inflammation in experimental glomerulonephritis in mice. *J Am Soc Nephrol*. 2007; 18:788–98. [PubMed: 17287428]
23. Reinecke H, Robey TE, Mignone JL, Muskheli V, Bornstein P, Murry CE. Lack of thrombospondin-2 reduces fibrosis and increases vascularity around cardiac cell grafts. *Cardiovasc Pathol*. 2013; 22:91–5. [PubMed: 22512900]
24. Amann K, Breitbach M, Ritz E, Mall G. Myocyte/capillary mismatch in the heart of uremic patients. *J Am Soc Nephrol*. 1998; 9:1018–22. [PubMed: 9621284]
25. Aoki J, Ikari Y, Nakajima H, Mori M, Sugimoto T, Hatori M, et al. Clinical and pathologic characteristics of dilated cardiomyopathy in hemodialysis patients. *Kidney Int*. 2005; 67:333–40. [PubMed: 15610259]
26. Zeisberg EM, Tarnavski O, Zeisberg M, Dorfman AL, McMullen JR, Gustafsson E, et al. Endothelial-to-mesenchymal transition contributes to cardiac fibrosis. *Nat Med*. 2007; 13:952–61. [PubMed: 17660828]
27. Kazakov A, Muller P, Jagoda P, Semenov A, Bohm M, Laufs U. Endothelial nitric oxide synthase of the bone marrow regulates myocardial hypertrophy, fibrosis, and angiogenesis. *Cardiovasc Res*. 2012; 93:397–405. [PubMed: 22106415]
28. Dhanabal M, Volk R, Ramchandran R, Simons M, Sukhatme VP. Cloning, expression, and in vitro activity of human endostatin. *Biochem Biophys Res Commun*. 1999; 258:345–52. [PubMed: 10329390]
29. Dhanabal M, Ramchandran R, Waterman MJ, Lu H, Knebelmann B, Segal M, et al. Endostatin induces endothelial cell apoptosis. *J Biol Chem*. 1999; 274:11721–6. [PubMed: 10206987]
30. Gu JW, Shparago M, Tan W, Bailey AP. Tissue endostatin correlates inversely with capillary network in rat heart and skeletal muscles. *Angiogenesis*. 2006; 9:93–9. [PubMed: 16791708]

Non Standard Abbreviations

ADMA asymmetric dimethyl arginine

ANG	angiotensin-2
CKD	chronic kidney disease
END	endostatin
EndMT	endothelial to mesenchymal transformation
ESRD	end stage renal disease
HCAEC	human coronary artery endothelial cells
NO	nitric oxide
TSP	thrombospondin-2

Highlights for Review

- Cell culture, human heart and blood, were studied to assess links of CKD and CVD
- Myocardial fibrosis increases and capillary supply are decreased in CKD
- Endothelial to mesenchymal transition (EndMT) is increased in CKD
- Circulating inhibitors of angiogenesis are increased in CKD
- EndMT and angiogenesis inhibitors may cause fibrosis and capillary loss in CKD

Figure 1. Evaluation of fibrosis and microvessels in the LV

(A) Pictures show representative left ventricular sections stained with Masson-Trichrome to visualize fibrotic tissue (stained in blue) (B) pictures shows immunohistochemical anti-CD31 staining to label microvessels (stained in brown) (A, B) left images shows heart tissue from an individual with preserved GFR, middle images from a patient with stage 3-4 CKD, and right images from a dialysis patient. (C-F) Quantitative analysis of Masson-Trichrome and anti-CD31 stained sections from n=21 preserved GFR, n=17 stage 3-4 CKD, and n=7

dialysis patients for (C) Fibrosis, (D) Microvascular density, (E) Myocyte size, and (F) Myocyte density.

Figure 2. EndMT in the heart: Confocal microscopy and qPCR expression analysis

(A) Representative confocal images of left ventricular tissue after immunofluorescent staining with antibodies for the fibroblast marker FSP1 (green) and endothelial cell marker CD31 (red). Nuclei are stained with DAPI (blue). Top row shows tissue from an individual with preserved renal function, middle row from a patient with stage 3-4 CKD, and the bottom row from a dialysis patient. Dual FSP1/CD31 positive cells, indicative of EndMT (arrows), increase with decreasing renal function. Magnification x63. (B-D) Quantitative analysis of n=11 preserved GFR, n=8 stage 3-4 CKD, and n=4 dialysis patients for (B) FSP+

cells/visual field, (C) FSP1/CD-31 double positive cells/visual field, indicative of cells undergoing EndMT. (D) Ratio of FSP1/CD31 double positive cells to all FSP+ cells, indicative of the fraction of EndMT-derived FSP1-positive fibroblasts. (E, F, G) Bar graphs show quantitative real time PCR for EndMT marker genes *SLUG* (E), *SNAIL* (F), and *TWIST* (G) in right atrial appendage of individuals with preserved function (n=3), with CKD stage 3-4 (n=3), and dialysis patients (n=3).

Figure 3. Effect of CKD serum on human coronary artery endothelial cells (HCAECs): proliferation, apoptosis, and EndMT

(A, B) Representative light microscopy images of HCAEC after 6 days of incubation with serum from subjects with preserved renal function (A) and with CKD (B). Medium with serum was changed every other day. Number of cells decreased upon CKD serum incubation and cell morphology of surviving cells changed towards more spindle shaped as compared to incubation with serum from an individual with preserved renal function. (C, D) HCAEC were incubated with serum from individuals with preserved renal function (n=30), or from CKD patients stage 2 (n=58), stage 3-4 (n=60), or stage 5 (n=14), and relative

apoptosis (as assessed by the Caspase-Glo 3/7 assay) and proliferation (as assessed by the WST-1 assay) were measured. (C) Relative proliferation of HCAECs decreased with more severe CKD. (D) Relative apoptosis of HCAEC increased with increasing CKD severity. (E-G) Bar graphs show quantitative real time PCR for EndMT marker genes *SLUG* (E), *SNAIL* (F), and *TWIST* (G) (all relative to *GAPDH*) in HCAEC upon incubation with healthy (n=3) versus CKD (n=9, 3 per stage) serum. Both *TWIST* and *SNAIL* are increased upon incubation with serum from CKD stages 3 through 5.

Figure 4. Serum concentrations of anti-angiogenic factors in CKD patients

Box plots show serum levels of (A) the NO-synthase inhibitor ADMA and (B-D) the angiogenesis inhibitors (B) Endostatin, (C) Thrombospondin-2 and (D) Angiopoietin-2. Boxes span the minimum and maximum values (log transformed). Horizontal lines represent the median. Vertical lines above and below each box encompass maximal and minimal values. Serum of individuals with preserved renal function (n= 30), or from CKD patients stage 2 (n=58), stage 3-4 (n=60), or stage 5 (n=14) was used for all of these measurements.

Figure 5. Effect of recombinant Angiopoietin-2, Thrombospondin-2, ADMA and Endostatin on proliferation, apoptosis and EndMT of HCAECs

HCAECs were treated with different concentrations of recombinant angiopoietin-2 (A-C), thrombospondin-2 (D-F), ADMA (G-I), or endostatin (J-L). Bar graphs show the effect of these factors on HCAEC proliferation (A, D, G, J), HCAEC apoptosis (B, E, H, K), and EndMT marker expression *SLUG* and *TWIST* (C, F, I, L).

Figure 6. Schematic for mechanisms of fibrosis and capillary rarefaction in CKD

High circulating concentrations of the angiogenesis inhibitors asymmetric dimethylarginine (ADMA), endostatin (END), angiopoietin-2 (ANG), and thrombospondin-2 (TSP) in CKD lead to EndMT causing microvascular rarefaction, fibroblast accumulation, and cardiac fibrosis.

Table 1
Baseline Characteristics of the Autopsy Cohort

Characteristic N (%)	Preserved Function (N=21)	Stage 3-4 CKD (N=17)	Dialysis (N=7)	P Value*
Demographics				
Age (years), mean $\pm$ SD	58.0 $\pm$ 15.2	72.8 $\pm$ 10.5	66.6 $\pm$ 5.6	0.003
Male sex	12 (57.1)	9 (52.9)	5 (71.4)	0.70
Race				0.33
White	16 (76.2)	13 (76.5)	3 (42.9)	
Black	3 (14.3)	3 (17.7)	3 (42.9)	
Labs (mean $\pm$ SD)				
eGFR, mL/min/1.73m ²	101.7 $\pm$ 34.2	37.6 $\pm$ 16.2	2.0 $\pm$ 0.0	<0.001
Serum creatinine, mg/dL	70.7 $\pm$ 26.5	203.3 $\pm$ 159.1	707.2 $\pm$ 0.0	<0.001
Medical History				
Diabetes	2 (9.5)	10 (58.8)	6 (85.7)	<0.001
Hypertension	16 (76.2)	14 (82.4)	7 (100.0)	0.36
Coronary disease	6 (28.6)	7 (41.2)	5 (71.4)	0.13
Myocardial infarction	4 (19.1)	7 (41.2)	4 (57.1)	0.12
Atrial fibrillation	3 (15.0)	5 (29.4)	0 (0.0)	0.21
Congestive heart failure	2 (9.5)	5 (29.4)	3 (42.9)	0.12
Obesity	5 (23.8)	5 (29.4)	2 (28.6)	0.92
Hyperlipidemia	7 (33.3)	12 (75.0)	5 (71.4)	0.03
History of cancer	1 (4.8)	3 (17.7)	0 (0.0)	0.26
Anemia	3 (15.0)	8 (53.3)	4 (57.1)	0.03
Past or present smoking	11 (61.1)	8 (50.0)	2 (28.6)	0.34
Number of diseased coronary arteries (mean $\pm$ SD) [#]	1.4 $\pm$ 1.5	1.7 $\pm$ 1.2	1.9 $\pm$ 1.2	0.71
Cause of Death				0.51
Infection	5 (23.8)	5 (29.4)	3 (42.9)	
Cardiovascular	6 (28.6)	8 (47.1)	2 (28.6)	
Other	10 (47.6)	4 (23.5)	2 (28.6)	
Medications				
Aspirin	5 (25.0)	10 (58.8)	6 (85.7)	0.01
ACE or ARB	6 (30.0)	9 (56.3)	3 (42.9)	0.28
Statin	7 (35.0)	9 (52.9)	5 (71.4)	0.22
Beta Blocker	10 (50.0)	10 (58.8)	5 (83.3)	0.35

S.D.=standard deviation. eGFR-estimated GFR.

* ANOVA and non-parametric trend test for normally and non-normally distributed continuous variables, respectively. χ^2 tests for count variables.

[#] Percent stenosis assessed pathologically was available for 17 patients with preserved function, 16 with Stage 3-4 CKD, and 7 on dialysis. Smoking status was missing in 4 individuals (preserved GFR-3, Stage 3-4 CKD-1). All dialysis patients were on hemodialysis.

Table 2
Histologic Findings

Characteristic Mean $\pm$ SD	Preserved Function-Stage 1 CKD	Stage 3-4 CKD	Dialysis	P _{trend}
Histology	(N=21)	(N=17)	(N=7)	
Fibrosis (%)	10.6 $\pm$ 4.8	11.9 $\pm$ 4.5	18.2 $\pm$ 6.6	0.003
Microvessels/field	459.1 $\pm$ 110.1	402.4 $\pm$ 69.7	386.9 $\pm$ 77.5	0.04
Cardiomyocytes/field	615.8 $\pm$ 214.3	573.2 $\pm$ 99.4	550.3 $\pm$ 121.9	0.31
Microvessel density (n/ μ M ²)	612.2 $\pm$ 146.7	536.6 $\pm$ 92.9	515.8 $\pm$ 103.3	0.04
Myocyte size (μ M ²)	623.3 $\pm$ 222.8	645.4 $\pm$ 197.4	595.4 $\pm$ 222.6	0.90
Myocyte density (n/ μ M ²)	821.0 $\pm$ 285.8	764.3 $\pm$ 132.5	733.8 $\pm$ 162.5	0.31
Vessels per myocyte	0.80 $\pm$ 0.19	0.73 $\pm$ 0.16	0.74 $\pm$ 0.18	0.24
Immunofluorescence	(N=11)	(N=8)	(N=4)	
FSP positive cells/field	4.2 $\pm$ 1.4	4.1 $\pm$ 2.0	6.6 $\pm$ 0.7	0.04
Double FSP/CD-31 positive cells/field	0.9 $\pm$ 0.7	1.4 $\pm$ 0.7	2.3 $\pm$ 1.3	0.01
Ratio of double positive to all FSP-positive cells	0.19 $\pm$ 0.12	0.36 $\pm$ 0.11	0.36 $\pm$ 0.21	0.02

SD=Standard deviation

Table 3

Baseline Characteristics of the Serologic Cohort

Characteristic N (%)	Preserved Function (N=30)	Stage 2 CKD (N=58)	Stage 3-4 CKD (N=60)	Stage 5 CKD (N=14)	P Value*
Demographics					
Age (years), mean $\pm$ SD	57.2 $\pm$ 8.3	61.1 $\pm$ 11.4	67.0 $\pm$ 11.8	48.9 $\pm$ 20.0	<0.001
Male sex	23 (76.7)	38 (65.5)	40 (66.7)	5 (35.7)	0.07
Race					0.01
White	26 (86.7)	45 (79.0)	46 (79.3)	5 (35.7)	
Black	3 (10.0)	7 (12.3)	5 (8.6)	6 (42.9)	
Other	1 (3.3)	5 (8.7)	7 (12.0)	3 (21.4)	
Labs and Physical Exam					
eGFR (mL/min/1.73m ²), mean $\pm$ SD	105.4 (11.8)	74.3 $\pm$ 8.0	38.5 $\pm$ 13.4	4.3 $\pm$ 4.3	<0.001
Serum creatinine (mg/dL), median (IQR)	70.7 (62.2 -79.5)	88.4 (79.6-106.1)	150.3 (123.8-221.0)	707.2 (707.2 -707.2)	<0.001
Systolic pressure (mm Hg), mean $\pm$ SD	128.1 $\pm$ 24.2	123.3 $\pm$ 19.8	132.8 $\pm$ 21.5	138.3 $\pm$ 21.0	0.05
Diastolic pressure (mm Hg), mean $\pm$ SD	73.7 $\pm$ 13.2	69.9 $\pm$ 11.9	69.6 $\pm$ 16.0	77.4 $\pm$ 18.7	0.25
Medical History					
Dialysis-dependent	0 (0.0)	0 (0.0)	0 (0.0)	10 (71.4)	<0.001
Diabetes	9 (30.0)	18 (31.0)	37 (61.7)	6 (42.9)	0.003
Hypertension	21 (70.0)	42 (72.4)	50 (83.3)	14 (100.0)	0.07
Myocardial infarction	6 (20.0)	16 (27.6)	14 (23.3)	4 (33.3)	0.77
Congestive heart failure	4 (13.3)	14 (24.1)	17 (28.3)	5 (35.7)	0.33
Obstructive lung disease	0 (0.0)	2 (3.5)	6 (10.0)	1 (14.3)	0.12
Peripheral vascular disease	2 (6.7)	3 (5.2)	11 (18.3)	2 (14.3)	0.11
Hyperlipidemia	22 (73.3)	41 (70.7)	42 (70.0)	10 (71.4)	0.99
Current smoking	4 (14.8)	6 (10.9)	2 (3.5)	1 (8.3)	0.30
Anemia	3 (10.3)	12 (21.4)	29 (50.9)	9 (64.3)	<0.001
Cause of CKD					<0.001
Other/unknown	--	--	39 (65.0)	3 (21.4)	
Diabetes	--	--	12 (20.0)	4 (28.6)	
Hypertension	--	--	7 (11.7)	2 (14.3)	
Glomerulonephritis	--	--	1 (1.7)	5 (35.7)	

Characteristic N (%)	Preserved Function (N=30)	Stage 2 CKD (N=58)	Stage 3-4 CKD (N=60)	Stage 5 CKD (N=14)	P Value*
Renal artery stenosis	--	--	1 (1.7)	0 (0.0)	
Medications					
Aspirin	25 (83.3)	43 (74.1)	44 (73.3)	7 (50.0)	0.14
ACE or ARB	15 (50.0)	29 (50.0)	37 (61.7)	10 (71.4)	0.33
Statin	23 (76.7)	38 (65.5)	45 (75.0)	8 (57.1)	0.39
Beta Blocker	18 (60.0)	38 (65.5)	45 (75.0)	8 (57.1)	0.38

SD=standard deviation, eGFR=estimated glomerular filtration rate, CKD=chronic kidney disease, IQR=interquartile range, ACE=angiotensin converting enzyme inhibitor, ARB=angiotensin receptor blocker, Race was missing in 1 participant, Smoking status was missing in 10%.

* ANOVA for continuous variables, Chi-squared for count variables.

Table 4

Factor Concentrations, HCAEC Apoptosis, and Proliferation

Factor, median (IQR)	Preserved Function (N=30)	Stage 2 CKD (N=58)	Stage 3-4 CKD (N=60)	Stage 5 CKD (N=14)	P Value*
Log Endostatin, (ng/mL)	4.5 ± 0.3	4.7 ± 0.5	5.4 ± 0.4 [†]	6.7 ± 0.4 [†]	<0.001
Log Thrombospondin-2, (pg/mL)	9.9 ± 0.6	9.9 ± 0.5	10.3 ± 0.6 [†]	10.1 ± 0.8	0.01
Log Angiopoietin-2 (ng/mL)	0.7 ± 0.6	0.7 ± 0.5	1.3 ± 0.6 [†]	1.9 ± 0.7 [†]	<0.001
Log ADMA (μmol/L)	-0.8 ± 0.2	-0.8 ± 0.2	-0.7 ± 0.2	-0.5 ± 0.3 [†]	<0.001
Log Apoptosis (relative units)	-1.2 ± 0.7	-1.0 ± 0.6	-1.1 ± 0.6	-0.6 ± 0.7 [†]	0.02
Proliferation (relative units) mean ± SD	1.2 ± 0.25	1.1 ± 0.23	0.99 ± 0.33 [†]	1.02 ± 0.25	0.03

S.D.=Standard deviation. I.Q.R.=Interquartile Range.

* ANOVA and non-parametric trend test for normally and non-normally distributed continuous variables, respectively.

[†] P<0.05 for comparison with preserved function by Tukey–Kramer post-hoc test.