The role of laser Doppler flowmetry tests, serum angiopoietin-2, asymmetric and symmetric dimethylarginine to predict outcome in chronic kidney disease

short title: Laser Doppler and serum biomarkers in CKD

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Abstract

Objective: The role of biochemical and functional markers of microvascular dysfunction to predict cardiovascular (CV) outcomes in non-dialyzed chronic kidney disease (CKD) remains unclear. In this prospective cohort study we assessed whether biochemical (serum level of angiopoietin-2 (Ang-2), asymmetric and symmetric dimethylarginin (ADMA, SDMA)) and functional (laser Doppler flowmetry (LDF)) measures of microvascular function predicted CV events, CV and all-cause mortality in CKD patients.

Methods: Post-occlusive reactive hyperemia area ($PORH_{HA}$), acetylcholine and sodium nitroprusside-mediated flow changes were estimated by LDF and Ang-2, ADMA and SDMA were assessed in 105 CKD patients at baseline. Multiple failure time Cox-regression analyses with backward elimination were performed to determine the predictors of the combined endpoint of CV mortality plus CV events or all-cause mortality plus CV events during a median of 66.6 (IQR 39.8-80.4) months of follow-up.

Results: In univariate models lnAng-2 and $lnPORH_{HA}$ both predicted the CV outcome besides age, diabetes, baseline CV disease, brachial pulse pressure and lnCRP. In multivariate analysis $lnPORH_{HA}$ (HR: 0.66 (95%CI: 0.49-0.89) per ln(mU*sec)), age (1.03 (1.01-1.06) per year), lnCRP (1.31 (1.06-1.64) per ln(mg/L)) and diabetes (3.33 (1.70-6.53)) remained significant predictors of the CV outcome while lnAng-2 did not enter the model. Neither of the microvascular variables were an independent predictor of all-cause mortality plus CV events.

Conclusions: Among the functional and biochemical microvascular parameters $PORH_{HA}$ seems to improve CV risk assessment in CKD. Nevertheless the robustness of traditional risk factors seems to outweigh the role of microvascular biomarkers on all-cause mortality plus CV events at this time.

Keywords: chronic kidney disease, laser Doppler flowmetry, angiopoietin-2, asymmetric dimethylarginine, symmetric dimethylarginine, prospective cohort study

Introduction

Cardiovascular (CV) disease is the major cause of morbidity and mortality in patients with chronic kidney disease (CKD)[1]. Furthermore, CV mortality in CKD patients is even more frequent than progression of renal failure to end-stage renal disease (ESRD)[2]. The development of CV disease in CKD patients involves the complex processes of progressive athero- and arteriosclerosis that includes microvascular dysfunction[3].

Following the discovery of the pivotal role of nitric oxide (NO) in endothelium-derived vascular relaxation by Furchgott and Zawadzki in 1980[4], different methods have been developed and used in laboratories for the early diagnosis of subclinical atherosclerosis and microvascular dysfunction. Only a few of these, however, such as the detection of minute amounts of albuminuria, increased high-sensitivity C-reactive protein levels or the measurement of carotid intima-media thickness have gained role in everyday practice[5]. Laser Doppler flowmetry (LDF) allows the non-invasive assessment of skin microvascular reactivity. Different tests have been developed for the evaluation of microcirculation with LDF. Using LDF with iontophoresis of acetylcholine and sodium nitroprusside or with the post-occlusive reactive hyperemia test (PORH) of the capillaries of the forearm we previously demonstrated endothelial dysfunction in patients with hypertension[6] and patients on dialysis[7].

Endothelial dysfunction could be estimated by different serum molecules that could also be linked to CV events in CKD, such as angiopoietin-2 (Ang-2), asymmetric dimethylarginin

(ADMA) or symmetric dimethylarginin (SDMA)[8-10]. Ang-2 functions as a dynamic autocrine negative regulator of the quiescent endothelium through inhibiting the endothelial receptor tyrosine kinase Tie-2-regulated signal transduction pathways[11]. Previously we found that Ang-2 increases with advanced CKD[12] and in a combined cohort of CKD patients stage 4 and 5, it was a predictor of mortality [8]. However, its predictive value for CV outcome on non-dialyzed CKD patients has never been tested.

ADMA and its isomer SDMA are endogenous products of methylated protein turnover that are present in the circulation in micromolar levels[13]. ADMA directly inhibits nitrogen-monoxide (NO) synthesis by competitively binding nitrogen-monoxide synthases (NOS), and both ADMA and SDMA may also reduce NO production by inhibiting the cellular uptake of L-arginine, a precursor of NO[13]. Both of these molecules are potential biomarkers of CV outcome in CKD, but their importance has not been conclusively clarified yet.

When a biological measure applies to be a marker of cardiovascular risk, among others, follow-up studies are also required to demonstrate its predictive value for different outcomes before their introduction into clinical practice [14]. While many groups used LDF to evaluate endothelial dysfunction, only one work is available in ESRD patients about its predictive value for cardiovascular mortality[15] and none in non-dialyzed CKD. Furthermore, neither the correlation, nor the predictive superiority of biomarkers vs. LDF to depict endothelial dysfunction and CV events has been shown in non-dialyzed CKD patients.

Therefore the aim of our study was to evaluate whether traditional risk factors, LDF parameters of iontophoresis and PORH were related to markers of microvascular dysfunction, such as Ang-2, ADMA and SDMA in stage 1-5 CKD non-dialyzed patients. We also aimed to study the prognostic importance of these parameters in our patients for CV events and CV or all-cause mortality. We hypothesized, that the functional and biochemical markers of

microvascular dysfunction would be associated with each other and that they would be predictors of CV events.

Materials and Methods

This was a prospective cohort study of hypertensive CKD patients with baseline cross-sectional analysis between the LDF parameters and biochemical markers of microvascular function, and analysis of these microvascular markers as predictors for CV events, CV- and all-cause mortality during follow-up. Initially 108 patients were enrolled but finally only 105 patients were followed-up and analyzed as three patients could not be contacted after the initial measurements.

Convenience sampling was used with consecutive inclusion of CKD patients presenting at two tertiary care nephrology outpatient clinics who were invited to participate in the study. None of the patients were hospitalized at the time of baseline investigations. No other specific exclusion criteria were applied. Antihypertensive treatment was tailored according to the latest recommendations of the European Society of Hypertension[16]. CKD patients in stage 1-5, non-dialyzed, who gave written informed consent for participation, were included and two appointments within the next two weeks for blood sampling and LDF measurements was arranged, Then patients were followed for a median of 66.6 months (interquartile range: 39.8-80.4). Follow-up data were collected between April 2007 and July 2014 by telephone interviews with the patients, their general practitioners or treating physicians, and the information gathered were in all cases verified by chart review. Follow-up was censored at the last occurrence of a documented CV event (acute coronary syndrome, heart failure requiring hospitalization, stroke or transient ischemic attack, peripheral artery disease verified by angiography or need for an intervention) or death due to the above CV events or any other causes. Laboratory data and vascular biomarkers were not collected during follow-up.

The protocol was approved by the Ethics Committees of the two hospitals that the outpatient clinics belonged to and was carried out in accordance with the tenets of the Declaration of Helsinki.

Epidemiologic and laboratory data

Baseline data on smoking habits (current), diabetes (DM, any type), hypertension, coronary artery disease (previous acute myocardial infarction or coronary intervention), chronic heart failure (previous diagnosis), peripheral artery disease (documented by angiography or intervention) and cerebrovascular disease (previous stroke or transient ischemic attack) were collected by health record review. Framingham 10-year cardiovascular risk scores were calculated for sensitivity analysis of the results[17].

Blood samples were taken after overnight fasting, between 7.00-8.00 a.m. On that morning regular medications were not taken, but there has been no medication withdrawal on previous days. Serum levels of hemoglobin, potassium, calcium, phosphorus, albumin, creatinine, C-reactive protein (CRP), cholesterol, were evaluated at baseline by a Hitachi auto-analyser. Intact parathormone 1-84 was determined by an immune-chemiluminometric two-site assay (CIBA-CORNING, Frenwald, Germany). Baseline eGFR was calculated using the four-variable Chronic Kidney Disease Epidemiology Collaboration equation. Albuminuria was characterised by albumin-to-creatinine ratio measured from first morning spot urine sample. Circulating serum Ang-2 concentrations were measured by an in-house immunoluminometric assay (ILMA) as previously reported by our group in details[18]. The coefficient of variation was <6%. The sensitivity threshold was 0.2 ng/ml. Assays were performed in duplicate by a single investigator blinded to patients' characteristics and outcome. Plasma ADMA and SDMA levels were assessed using high-performance liquid chromatography-tandem mass spectrometry[19].

Laser Doppler flowmetry measurements

LDF measurements were performed at the second appointment between 10-12 a.m. Patients had a non-standardized light breakfast and took their regular medications 3 hours before the LDF measurements. Patients were asked to refrain from smoking on the day of the study and not to consume caffeine-containing drinks at least 4 hours before the start of the measurements. LDF measurements and blood sampling were done on separate days within a week.

LDF measurements were carried out in a temperature-controlled room (24±1°C). Upon arrival and a 5-minute rest, two consecutive brachial blood pressure measurements were taken one minute apart on each arm in the sitting position with a validated BpTru device (VSM Medtech, Vancouver, Canada). The mean value was calculated for each arm, and the higher of these was further taken as brachial systolic and diastolic blood pressures and heart rate. Then subjects were set in the supine position for a 20-minute acclimatization period. Before starting the LDF measurements, the flexor surface of the right forearm was gently cleansed using alcohol solution.

The laser Doppler instrument Periflux 5001 (wavelength 780 mm) and the micropharmacology system PeriIont was used during the study. The same iontophoresis protocols were used as in our previous studies[6, 7]. The drug delivery electrode was filled with 140 µl acetylcholine 1% (Clinalfa AG, Switzerland) and was attached with the laser probe to the volar surface of the right forearm. The position of the probe was chosen in order to avoid hair, freckles and broken skin. The dispersive electrode was attached to the volar aspect of the wrist to complete the circuit. We placed a control standard probe 4 cm laterally from the drug delivery electrode. After registration of the baseline flow (60 s) two doses of acetylcholine were delivered using an anodal current (0.1mA for 30 s and 0.16mA for 30 s)

with a 120 s interval. Using a new delivery electrode two doses of sodium nitroprusside 1% (Nitropress, ABBOTT, USA) were delivered using a catodal current (0.1mA for 20 s and 0.1mA for 30 s) with a 120 s interval.

During the postocclusive reactive hyperemia (PORH) test after the registration of the baseline flow (60 s) arterial occlusion was performed with a suprasystolic pressure using the pneumatic cuff of a sphygmomanometer for 3 min (biological zero), then after the release of the pressure we measured the skin hyperemia on the volar surface of the left forearm 10 cm below the elbow with a standard laser Doppler probe. Another standard probe was put on the skin of the right forearm as a control.

About the principles of LDF measurements we also refer to our previous publications[6, 7]. The LDF output is semi-quantitative and is expressed in perfusion units (PU) of output voltage (1 PU = 10 mV) in accordance with the general consensus (European Laser Doppler Users Groups, London 1992). Because the output is not easily translated into absolute values of blood flow, in case of iontophoresis, the magnitude of the change in skin perfusion after the second administered dose of acetylcholine or sodium nitroprusside was calculated as the ratio between peak and baseline perfusion and expressed as percent of baseline (ACh, SNP values). In case of PORH, the software analyzed the data automatically and calculated several indexes such as the initial baseline value, slope value, peak flow, percent change in perfusion from baseline to maximum values, time to reach the maximum hyperemia, time to reach the half value after the maximum hyperemia, and the area of hyperemia. This latter measure (abbreviated as: $PORH_{HA}$, unit: PU*sec – perfusion unit * second) seems to be the most accurate parameter to assess the hyperemic response, as it includes three variables (speed, intensity, and duration) and this was used in the analyses as representative of the microvascular function of PORH[20, 21].

According to previous measurements in our laboratory[6], the day to day variability of this system was 16-21%, which is comparable to other studies[21] and to the wildly accepted brachial artery flow-mediated vasodilation technique (17.5%)[22]. The LDF measurements were performed and analyzed by the same examiners (JN, JE, GG, LS) throughout the study.

Statistical analysis

All data analysis was performed by STATA IC version 10.0 (StataCorp Lp. Texas USA) and Statistica version 11.0 (StatSoft Inc. Tulsa USA). Continuous data are given as mean and standard deviation, or in case of evidence against normal distribution, as median and interquartile range. For further analyses non-normally distributed data were transformed logarithmically. All those variables that were log-transformed had positively skewed distribution that improved after the transformation visually although not all satisfied tests for normality. Thus in the statistical modeling described in the next paragraphs, log-transformed and non-log transformed variables were included in the models simultaneously. In the group comparisons of anthropometric and clinical parameters Student's t-test for independent samples, Mann Whitney U tests and chi-squared tests were used, as appropriate.

In the baseline cross-sectional analyses first Spearman correlations were run between the six microvascular parameters (ACh, SNP, $PORH_{HA}$, Ang-2, ADMA, SDMA). Then, univariate and multivariate linear regressions were performed to determine associations of these six microvascular parameters with baseline clinical variables and used medications. The predictor clinical variables considered were the ones listed in table 1. The variables that showed a significant association with the given microvascular variable in univariate models were considered in the final multivariate model.

The primary outcome of the study was the occurrence of the combined endpoint of CV events and CV mortality or CV events and all-cause mortality, as defined above.

To assess the predictive values of ACh, SNP, $PORH_{HA}$, Ang-2, ADMA and SDMA for the primary outcome multiple failure times Cox proportional hazard regression analyses were used with conditional risk set modeling. This method accommodates for the fact that one patient may have had more than one event during follow up. We first performed univariate analyses considering variables listed in Table 1 and all groups of medications. Confounding was addressed in multivariate Cox-regression models with adjustment for potential clinical predictors. In case of medications those groups were considered that were independently related to the three LDF or three biochemical parameters listed in table 3 or those that showed a univariate association with the outcomes ($p<0.1$). In the final multivariate models instead of the use of separate variables for "oral antidiabetic drugs" and "insulin", the variable "diabetes" was used, as these two groups did not improve the Cox-models. Final models were selected using backward elimination to reach the most parsimonious models. As a sensitivity analysis we repeated these analyses using the other endpoint (CV events plus all-cause mortality) as the outcome. For further sensitivity analysis area under the receiver-operator characteristics curves were compared and net reclassification improvement (NRI) was calculated from the probability scores of logistic regression models using the Framingham cardiovascular risk score and $lnPORH_{HA}$ as predictors and both primary outcomes as dependent variables[23].

A p-value with a two-sided alpha of <0.05 was considered statistically significant. Hazard ratios are presented with their corresponding 95% confidence intervals.

Results

We report data on 105 patients with CKD at the two centers who had follow-up data. Baseline clinical, laboratory, hemodynamic and LDF data of the patients are presented in table 1, comparing those with an eGFR less than or equal to those with higher than the median 35

ml/min/1.73m^2. Diabetic (27.6%) and hypertensive nephropathy (18.1%), tubulointerstitial disease (18.1%) and glomerulonephritis (14.1%) were the most frequent causes of CKD. Almost all patients took an ACE-inhibitor or angiotensin receptor blocker (88.6%), many of them a diuretic (74.3%), calcium channel blocker (53.3%) or beta-blocker (54.2%), less alpha-blocker (18.1%) and centrally effective antihypertensive drugs (13.3%). Sixty-two percent of the patients were on statins, 22.8% on oral antidiabetics, 21% on insulin, 14.3% on nitrate and 12.4% on fibrate therapy. Half of the patients took any kind of antiplatelet therapy. The group with an eGFR below 35 ml/min/1.73 m^2 had a worse metabolic status as indicated by their elevated phosphorus, parathyroid hormone, CRP, urinary albumin-to-creatinine ratio and lower hemoglobin values. No significant differences were found between these two groups in respect of their ACh, SNP or $PORH_{HA}$ values. Ang-2, ADMA and SDMA levels, however, were significantly higher in those with more advanced CKD.

The second table presents Spearman correlation coefficients between different LDF and serum microvascular biomarkers. There were significant baseline correlations among the three biochemical parameters and also among the three LDF parameters, but not between the biochemical and LDF measures. The third table shows independent associations of the six vascular parameters with other measures at baseline, based on multivariate linear regression analysis. The highest R^2 was found for lnSDMA mainly due to its strong relation to eGFR. Overall however, the model R^2 values for the 6 parameters were small or moderate.

Outcome status was available for all the 105 patients at the end of the follow-up. By that time a total of 50 primary outcome events occurred in 38 patients. It represents an incidence rate of CV diseases of 8.5 events per 100 patient-years in our population. Sixteen patients died of CV causes (acute coronary syndrome n=4, stroke n=3, heart failure n=8, and peripheral artery disease n=1), and there were 34 additional CV events (acute coronary syndrome n=8, stroke n=6, heart failure n=13, peripheral artery disease n=7). During follow-up 12 patients died of

non-CV causes (senile dementia n=5, pneumonia n=2, accidents n=2, lung cancer n=1, colorectal cancer n=1, pancreatitis n=1).

The fourth table summarizes the results of the uni-and multivariate Cox proportional hazard regression analyses for all microvascular LDF and biochemical parameters and for those other baseline variables that showed a significant association with the endpoints. In univariate models, only $lnPORH_{HA}$ and lnAng-2, but not the other four baseline microvascular parameters, were significantly related to these two outcomes. In multivariate analyses $lnPORH_{HA}$ remained an independent predictor of CV mortality and CV events besides age, CRP and diabetes. While LnAng-2 was a significant predictor of this outcome (CV mortality and CV events) independent of age, sex, and baseline CV disease, but once diabetes or eGFR was introduced into the model it lost its statistical significance. When all-cause mortality was introduced instead of CV mortality as outcome even $lnPORH_{HA}$ lost its predictive value leaving age, diabetes pulse pressure and the use of fibrates as independent risk factors.

Based on multivariate logistic regressions using the Framingham 10-year CV risk score and the Framingham score plus $lnPORH_{HA}$ as predictors, both parameters were significantly related to CV events plus CV mortality (1.07 (1.03-1.11) and 0.66 (0.43-1), respectively). The Framingham risk score had an overall good discrimination (AUC: 0.75 SE: 0,05) however the addition of $lnPORH_{HA}$ did not further improve discrimination (AUC: 0.76 SE: 0.05, p=0.54). In contrast, the NRI showed a significant reclassification improvement of 21.8% (SE 0.09) using three risk categories (0-19.9, 20-44.9, ≥45%). However, only Framingham score but not $lnPORH_{HA}$ was an independent predictor of all-cause mortality plus CV events (1.09 (1.04-1.14) and 0.75 (0.49-1.14), respectively).

Discussion

The major finding of our cohort study is that among the tested functional (ACh, SNP, $PORH_{HA}$) and biochemical (Ang-2, ADMA, SDMA) microvascular parameters $PORH_{HA}$ independently predicted CV events and CV mortality in non-dialyzed CKD patients. Nevertheless the robustness of traditional risk factors seems to outweigh the predictive role of this microvascular biomarker when all-cause mortality and incidence of CV events are considered.

The different LDF methods of iontophoresis have frequently been studied in pathological conditions. Acetylcholine is assumed to cause endothelial-dependent, while sodium nitroprusside endothelium-independent vasodilation[24]. Later studies however showed, that the cutaneous blood flow increase induced by acetylcholine administration is also influenced by axon reflex and prostanoids as well[25], suggesting that the acetylcholine-induced reactive hyperemia has also endothelium-independent components. When compared to our previous studies, the magnitude of the evoked hyperemia after acetylcholine and sodium nitroprusside iontophoresis in this work was between those who had only hypertension and those who were dialyzed, and lower compared to healthy controls[7]. Surprisingly however, no difference was found between those with less and more advanced CKD in their acetylcholine or sodium nitroprusside responses, and eGFR was not an independent determinant of these parameters. These findings are in agreement with a recent publication, where Thang et al.[26] demonstrated that in patients with advanced CKD reactive hyperemia for acetylcholine and sodium nitroprusside iontophoresis were not associated with eGFR.

At baseline both acetylcholine and sodium nitroprusside responses were inversely related to the presence of diabetes. This is in line with the literature as Beer et al.[27] have previously shown that diabetes significantly influenced cutaneous vascular response to acetylcholine and sodium nitroprusside iontophoresis. Moreover, Brooks et al.[28] found a progressive fall of both acetylcholine and sodium nitroprusside-induced microvascular responsiveness in

parallel with the development of microvascular complications of diabetes. The use of antiplatelet medication and diuretics were both independently and negatively associated with sodium nitroprusside-evoked hyperemia in our study. We suppose that these associations do not reflect a direct effect, but their use is a sign of the presence of more severe comorbidities, associated with lower overall microvascular reactivity.

From the different PORH parameters we have chosen hyperemia area ($PORH_{HA}$), as it reflects speed, intensity, and duration of the hyperemic response and previously it was associated with the development of coronary heart disease in ESRD patients [15, 20]. We, however, found no significant difference in $PORH_{HA}$ between those with less and more advanced CKD, and eGFR was not an independent determinant of $PORH_{HA}$. This, together with the results of iontophoresis, suggests that endothelial dysfunction evaluated by the different LDF tests is an early process, and that the progression of chronic kidney disease per se does not influence its severity significantly, until dialysis becomes necessary. On the grander scheme of using PORH in CKD patients for the assessment and evaluation of cardiovascular risk, we think if other studies confirm our findings, $PORH_{HA}$ might be a recommended for risk stratification in all stages of CKD.

$PORH_{HA}$, however, was inversely and significantly related to brachial pulse pressure which confirms the known link between micro-and macrovascular function[29]. Stiff central arteries cause high pulsatility of central aortic pressure, resulting high transmission of pulsatile energy into the periphery, which is thought to promote microvascular injury [30]. $PORH_{HA}$ was also inversely associated with the use of calcium channel blockers and fibrates. As both of these groups of medications were found to be beneficial for vasodilatory responses measured with flow-mediated vasodilation [31-33], we believe, these associations do not refer to a direct effect, rather the severity of comorbidities of patients.

There were significant baseline correlations among the three biochemical parameters of microvascular dysfunction and also among the three laser Doppler parameters, but not between the biochemical and laser Doppler measures. This finding suggests first, that the measured parameters have their internal validity, and second, that the biochemical parameters provide information on vessel function that is different from what laser Doppler functional tests provide.

Among the studied LDF parameters only $PORH_{HA}$, but not ACh or SNP, was found to be a predictor of CV mortality and CV events. The predictive role of acetylcholine and sodium nitroprusside iontophoresis for CV events or mortality has not been studied previously[34]. While our current data question the clinical use of iontophoresis for risk prediction in CKD, more confirmatory follow-up studies are needed in different populations before one may conclude that iontophoresis should be reserved for the laboratory use.

The predictive role of $PORH_{HA}$ in our patient population is in line with the findings of Kruger et al, who demonstrated in ESRD patients that development of coronary artery disease was associated with the postocclusive recruitment of dermal capillaries[15]. While our data are the first to show an independent predictive value of $PORH_{HA}$ for CV mortality and CV events, even $PORH_{HA}$ lost its independent predictive role when all-cause mortality and CV events were considered as outcome. This is perhaps due to the robust effects of classical risk factors, such as age, diabetes or brachial pulse pressure, and also due to the difference in predictors of CV and all-cause mortality. The significant NRI means that $PORH_{HA}$ improved prediction of CV risk over the Framingham scores. It seems therefore that this simple LDF test is worth of further study and bears the promise of clinical application.

Interestingly, in the final multivariate Cox-proportional regression analysis fibrate use was an independent predictor of CV events plus all-cause mortality. As only 12.4% of our 105

patients took fibrates and we had no follow-up data on medication use, this finding requires cautious and critical interpretation. Although fibrates can increase creatinin, the safety of their use above GFR 30 ml/min was confirmed by the huge, randomized FIELD study [35].

Regarding to the studied serum markers, previously we found that Ang-2 increases with advanced CKD[12]. In line with this observation, in our current cohort Ang-2 was also elevated in more advanced CKD, although in multivariate analysis eGFR failed to remain an independent determinant of Ang-2. Whether the significant association between phosphorus and Ang-2 is only the consequence of decreased kidney function, or it represents yet another facet of mineral bone disease, remains to be investigated. Another independent determinant of Ang-2 level was CRP, which was also found to be associated with Ang-2 in coronary heart disease patients[36]. Ang-2, via the loss of Tie-2 signaling, destabilizes the endothelium and facilitates inflammatory response to cytokines[37], and this may be an explanation for this association. Finally, Ang-2 was inversely related to serum cholesterol level. The explanation for this observation is not clear so far, but this finding is in line with our previous study, where the same inverse relationship was found in more advanced CKD[12].

While in univariate analysis Ang-2 was a predictor of both outcomes, in multivariate analysis Ang-2 failed to remain an independent risk factor. This is in contrast with our previous study, where Ang-2 was found to predict mortality in CKD patients independent of other risk factors[8]. Perhaps the less severe renal failure among our current population may explain for these contradictory results.

Regarding to ADMA and SDMA, the other two biochemical markers measured in our study, we found their elevated levels in those with more advanced CKD, which is in line with the literature data[9, 38]. While ADMA was found to be the predictor of mortality or CV events in ESRD previously, as well as in patients with advanced CKD[9, 39, 40], in our current study

neither ADMA nor SDMA predicted the outcomes. We assume that differences in the patient population or the used medications may have led to this conflicting finding. Almost all of our patients had hypertension (98%) and most took RAAS (renin-angiotensin-aldosterone system) inhibitors (88.6%). Previously RAAS inhibitors were found to reduce serum ADMA levels and that the use of RAAS inhibitors was an independent predictor of arginine/ADMA ratio[41, 42]. Although no medication was an independent predictor of ADMA in our study, the use of RAAS inhibitors had the highest point estimate among medications (β:-0.130, p=0.138). Moreover, the use of RAAS inhibitors was an independent negative predictor of SDMA. The ratio of patients who took RAAS inhibitors was much lower in the study of Hov et al (74%)[40, 41] and Lu et al (34%)[9]. Moreover, our patients were younger compared to those of Lu et al[9]. Age was an independent predictor of ADMA level in our study and in the study of Hov et al as well[41]. Furthermore, diabetes was more frequent (47%) among our patients compared to the study of Hov et al[40, 41]. Interestingly, in the large population-based study of Böger et al[43], in patients with diabetes ADMA failed to predict mortality. The same authors hypothesized, that this phenomenon may be explained by the observed uncoupling of endothelial nitric oxide synthase (eNOS) in diabetes[44]. The activity of uncoupled eNOS can lead to excessive superoxide production which can have detrimental effects directly or by reacting with vascular NO to form the highly reactive peroxynitrite. In this condition the inhibition of the uncoupled eNOS by ADMA or SDMA can have an unexpected beneficial effect and might modify the survival of patients[44]. This hypothesis however has never been tested in diabetic CKD patients.

A limitation of our study is the relatively low number of patients involved; however this is counterbalanced by the long follow-up period and by the use of multiple failure time analysis both of which likely decreased the chance of a beta-error. Given the relatively low number of participants and outcome events, we decided to use a stepwise elimination of potential co-

variates (even those that may be physiologically connected)), in line with the hypothesis generating purpose of our study. According to a post-hoc power analysis, given the mean values and SD observed for lnPORH in participants with and without an outcome (5.83, 6.38, 1.21, respectively), an alpha of 0.05, we had a 69% power to detect the observed difference. Our sophisticated analysis however probably provided somewhat larger power than the calculated as it took into account time to the event and also multiple events within participants. Another limitation comes from the convenience sampling with consecutive enrollment of patients in the two tertiary care nephrology clinics, and therefore selection bias that may limit generalizability (i.e. high baseline CV disease risk burden of our patients) cannot be ruled out.

In conclusions, among the studied LDF and biochemical microvascular parameters only $PORH_{HA}$ predicted CV events plus CV mortality, although even $PORH_{HA}$ lost its statistical significance when all-cause mortality plus CV events were considered as outcome. Although the robustness of traditional, Framingham risk factors seems to outweigh the predictive role of microvascular biomarkers on all-cause mortality plus CV events, the latter seems to be an additional marker to improve CV risk assessment in CKD. Our study confirms the role of age, diabetes or brachial pulse pressure on clinical outcome prediction in CKD patients that may further be improved by the evaluation of this simple laser-Doppler parameter.

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Conflict of interests

None.

References

1. Sarnak MJ, Levey AS, Schoolwerth AC, Coresh J, Culleton B, Hamm LL, et al. Kidney disease as a risk factor for development of cardiovascular disease: a statement from the American Heart Association Councils on Kidney in Cardiovascular Disease, High Blood Pressure Research, Clinical Cardiology, and Epidemiology and Prevention. Circulation. 2003; 108 (17):2154-69.

2. Tonelli M, Wiebe N, Culleton B, House A, Rabbat C, Fok M, et al. Chronic kidney disease and mortality risk: a systematic review. Journal of the American Society of Nephrology : JASN. 2006; 17 (7):2034-47.

3. Trappenburg MC, van Schilfgaarde M, Frerichs FC, Spronk HM, ten Cate H, de Fijter CW, et al. Chronic renal failure is accompanied by endothelial activation and a large increase in microparticle numbers with reduced procoagulant capacity. Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association. 2012; 27 (4):1446-53.

4. Furchgott RF, Zawadzki JV. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature. 1980; 288 (5789):373-6.

5. Perk J, De Backer G, Gohlke H, Graham I, Reiner Z, Verschuren M, et al. European Guidelines on cardiovascular disease prevention in clinical practice (version 2012). The Fifth Joint Task Force of the European Society of Cardiology and Other Societies on Cardiovascular Disease Prevention in Clinical Practice (constituted by representatives of nine societies and by invited experts). Developed with the special contribution of the European

Association for Cardiovascular Prevention & Rehabilitation (EACPR). European heart journal. 2012; 33 (13):1635-701.

6. Farkas K, Kolossvary E, Jarai Z, Nemcsik J, Farsang C. Non-invasive assessment of microvascular endothelial function by laser Doppler flowmetry in patients with essential hypertension. Atherosclerosis. 2004; 173 (1):97-102.

7. Farkas K, Nemcsik J, Kolossvary E, Jarai Z, Nadory E, Farsang C, et al. Impairment of skin microvascular reactivity in hypertension and uraemia. Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association. 2005; 20 (9):1821-7.

8. David S, John SG, Jefferies HJ, Sigrist MK, Kumpers P, Kielstein JT, et al. Angiopoietin-2 levels predict mortality in CKD patients. Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association. 2012; 27 (5):1867-72.

9. Lu TM, Chung MY, Lin CC, Hsu CP, Lin SJ. Asymmetric dimethylarginine and clinical outcomes in chronic kidney disease. Clinical journal of the American Society of Nephrology : CJASN. 2011; 6 (7):1566-72.

10. Memon L, Spasojevic-Kalimanovska V, Bogavac-Stanojevic N, Kotur-Stevuljevic J, Simic-Ogrizovic S, Giga V, et al. Assessment of endothelial dysfunction: the role of symmetrical dimethylarginine and proinflammatory markers in chronic kidney disease and renal transplant recipients. Disease markers. 2013; 35 (3):173-80.

11. Scharpfenecker M, Fiedler U, Reiss Y, Augustin HG. The Tie-2 ligand angiopoietin-2 destabilizes quiescent endothelium through an internal autocrine loop mechanism. Journal of cell science. 2005; 118 (Pt 4):771-80.

12. 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.

Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association. 2010; 25 (8):2571-6.

13. Schwedhelm E, Boger RH. The role of asymmetric and symmetric dimethylarginines in renal disease. Nature reviews Nephrology. 2011; 7 (5):275-85.

14. Hlatky MA, Greenland P, Arnett DK, Ballantyne CM, Criqui MH, Elkind MS, et al. Criteria for evaluation of novel markers of cardiovascular risk: a scientific statement from the American Heart Association. Circulation. 2009; 119 (17):2408-16.

15. Kruger A, Stewart J, Sahityani R, O'Riordan E, Thompson C, Adler S, et al. Laser Doppler flowmetry detection of endothelial dysfunction in end-stage renal disease patients: correlation with cardiovascular risk. Kidney international. 2006; 70 (1):157-64.

16. Mancia G, De Backer G, Dominiczak A, Cifkova R, Fagard R, Germano G, et al. 2007 Guidelines for the management of arterial hypertension: The Task Force for the Management of Arterial Hypertension of the European Society of Hypertension (ESH) and of the European Society of Cardiology (ESC). European heart journal. 2007; 28 (12):1462-536.

17. D'Agostino RB, Sr., Vasan RS, Pencina MJ, Wolf PA, Cobain M, Massaro JM, et al. General cardiovascular risk profile for use in primary care: the Framingham Heart Study. Circulation. 2008; 117 (6):743-53.

18. Lukasz A, Hellpap J, Horn R, Kielstein JT, David S, Haller H, et al. Circulating angiopoietin-1 and angiopoietin-2 in critically ill patients: development and clinical application of two new immunoassays. Critical care (London, England). 2008; 12 (4):R94.

19. Martens-Lobenhoffer J, Bode-Boger SM. Fast and efficient determination of arginine, symmetric dimethylarginine, and asymmetric dimethylarginine in biological fluids by hydrophilic-interaction liquid chromatography-electrospray tandem mass spectrometry. Clinical chemistry. 2006; 52 (3):488-93.

20. Stiefel P, Moreno-Luna R, Vallejo-Vaz AJ, Beltran LM, Costa A, Gomez L, et al. Which parameter is better to define endothelial dysfunction in a test of postocclusive hyperemia measured by laser-Doppler flowmetry? Coronary artery disease. 2012; 23 (1):57-61.

21. Kubli S, Waeber B, Dalle-Ave A, Feihl F. Reproducibility of laser Doppler imaging of skin blood flow as a tool to assess endothelial function. Journal of cardiovascular pharmacology. 2000; 36 (5):640-8.

22. van Mil AC, Greyling A, Zock PL, Geleijnse JM, Hopman MT, Mensink RP, et al. Impact of volunteer-related and methodology-related factors on the reproducibility of brachial artery flow-mediated vasodilation: analysis of 672 individual repeated measurements. Journal of hypertension. 2016; 34 (9):1738-45.

23. Sundstrom J, Byberg L, Gedeborg R, Michaelsson K, Berglund L. Useful tests of usefulness of new risk factors: tools for assessing reclassification and discrimination. Scandinavian journal of public health. 2011; 39 (4):439-41.

24. Newton DJ, Khan F, Belch JJ. Assessment of microvascular endothelial function in human skin. Clinical science (London, England : 1979). 2001; 101 (6):567-72.

25. Cracowski JL, Minson CT, Salvat-Melis M, Halliwill JR. Methodological issues in the assessment of skin microvascular endothelial function in humans. Trends in pharmacological sciences. 2006; 27 (9):503-8.

26. Thang OH, Serne EH, Grooteman MP, Smulders YM, Ter Wee PM, Tangelder GJ, et al. Premature aging of the microcirculation in patients with advanced chronic kidney disease. Nephron extra. 2012; 2 (1):283-92.

27. Beer S, Feihl F, Ruiz J, Juhan-Vague I, Aillaud MF, Wetzel SG, et al. Comparison of skin microvascular reactivity with hemostatic markers of endothelial dysfunction and damage in type 2 diabetes. Vascular health and risk management. 2008; 4 (6):1449-58.

28. Brooks BA, McLennan SV, Twigg SM, Yue DK. Detection and characterisation of microcirculatory abnormalities in the skin of diabetic patients with microvascular complications. Diabetes & vascular disease research : official journal of the International Society of Diabetes and Vascular Disease. 2008; 5 (1):30-5.

29. Oliver JJ, Webb DJ. Noninvasive assessment of arterial stiffness and risk of atherosclerotic events. Arteriosclerosis, thrombosis, and vascular biology. 2003; 23 (4):554-66.

30. Feihl F, Liaudet L, Waeber B. The macrocirculation and microcirculation of hypertension. Current hypertension reports. 2009; 11 (3):182-9.

31. Muiesan ML, Salvetti M, Belotti E, Paini A, Rosei CA, Aggiusti C, et al. Effects of barnidipine in comparison with hydrochlorothiazide on endothelial function, as assessed by flow mediated vasodilatation in hypertensive patients. Blood pressure. 2011; 20 (4):244-51.

32. Wei D, He WY, Lv QZ. Effect of nisoldipine and olmesartan on endothelium-dependent vasodilation in essential hypertensive patients. CNS neuroscience & therapeutics. 2012; 18 (5):400-5.

33. Harmer JA, Keech AC, Veillard AS, Skilton MR, Marwick TH, Watts GF, et al. Fenofibrate effects on arterial endothelial function in adults with type 2 diabetes mellitus: A FIELD substudy. Atherosclerosis. 2015; 242 (1):295-302.

34. Babos L, Jarai Z, Nemcsik J. Evaluation of microvascular reactivity with laser Doppler flowmetry in chronic kidney disease. World journal of nephrology. 2013; 2 (3):77-83.

35. Ting RD, Keech AC, Drury PL, Donoghoe MW, Hedley J, Jenkins AJ, et al. Benefits and safety of long-term fenofibrate therapy in people with type 2 diabetes and renal impairment: the FIELD Study. Diabetes care. 2012; 35 (2):218-25.

36. Wang X, Yong H, Mi L, Bai Y, Guo L, Gao W, et al. Changes and significance of serum angiopoietin-2 levels in patients with coronary heart disease. Biomarkers : biochemical indicators of exposure, response, and susceptibility to chemicals. 2012; 17 (8):745-9.

37. Fiedler U, Reiss Y, Scharpfenecker M, Grunow V, Koidl S, Thurston G, et al. Angiopoietin-2 sensitizes endothelial cells to TNF-alpha and has a crucial role in the induction of inflammation. Nature medicine. 2006; 12 (2):235-9.

38. Tutarel O, Denecke A, Bode-Boger SM, Martens-Lobenhoffer J, Schieffer B, Westhoff-Bleck M, et al. Symmetrical dimethylarginine outperforms CKD-EPI and MDRD-derived eGFR for the assessment of renal function in patients with adult congenital heart disease. Kidney & blood pressure research. 2011; 34 (1):41-5.

39. 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 (9299):2113-7.

40. Hov GG, Aasarod KI, Sagen E, Asberg A. Arginine, dimethylated arginine and homoarginine in relation to cardiovascular risk in patients with moderate chronic kidney disease. Clinical biochemistry. 2015.

41. Hov GG, Sagen E, Hatlen G, Bigonah A, Asberg A, Aasarod K. Arginine/asymmetric dimethylarginine ratio and cardiovascular risk factors in patients with predialytic chronic kidney disease. Clinical biochemistry. 2011; 44 (8-9):642-6.

42. Celik T, Iyisoy A, Yuksel C, Jata B. The beneficial effects of angiotensin-converting enzyme inhibitors on serum asymmetric dimethylarginine levels in the patients with cardiovascular disease. International journal of cardiology. 2010; 142 (1):107-9.

43. Boger RH, Sullivan LM, Schwedhelm E, Wang TJ, Maas R, Benjamin EJ, et al. Plasma asymmetric dimethylarginine and incidence of cardiovascular disease and death in the community. Circulation. 2009; 119 (12):1592-600.

44. Anderssohn M, Schwedhelm E, Luneburg N, Vasan RS, Boger RH. Asymmetric dimethylarginine as a mediator of vascular dysfunction and a marker of cardiovascular disease and mortality: an intriguing interaction with diabetes mellitus. Diabetes & vascular disease research : official journal of the International Society of Diabetes and Vascular Disease. 2010; 7 (2):105-18.

Table 1. Baseline demographic, clinical, hemodynamic and laboratory characteristics and laser-Doppler test results for all participants and by eGFR status (over or below the median).

variable	**all patients**	**>35**	**≤35**		**variable**	**all patients**	**>35**	**≤35**
n	105	53	52		Cholesterol (mmol/l)	4.88 (1.01)	4.90 (1.10)	4.84 (1.11)
Age (year)	65 (13.1)	64.1 (13.7)	65.9 (12.6)		Potassium (mmol/l)	4.59 (0.55)	4.51 (0.51)	4.65 (0.59)
Male % (n)	49 (51)	52 (28)	44 (23)		Calcium (mmol/l)	2.36 (0.12)	2.35 (0.10)	2.37 (0.14)
BMI	28.2 (5.0)	27.8 (4.7)	28.7 (5.3)		Phosphorus (mmol/l)	1.22 (0.24)	1.14 (0.20)	**1.30 (0.24)** [#]
Current smoker % (n)	11 (12)	11 (6)	11 (6)		PTH* (pg/ml)	56 (38-102)	45 (25-58)	**92 (54-168)**[#]
Baseline CV disease % (n)	62 (65)	55 (29)	69 (36)		Albumin (g/l)	45.2 (4.2)	45.0 (4.4)	45.5 (4.1)
Diabetes % (n)	45 (47)	38 (20)	52 (27)		CRP* (mg/l)	2.3 (0.9-4.5)	1.60 (0.7-3.1)	**3.05 (1.20-6.25)**[#]
Framingham score*	21.4 (13-32.6)	23.1 (12.6-32.7)	20.3 (13.6-32.5)		ACR* (mg/mmol)	7.2 (1.7-46)	4.6 (0.98-28.8)	**16.7 (3.9-83.1)**[#]
SBP (mmHg)	134 (15.8)	135 (16.2)	134 (15.6)		Ang-2*(ng/ml)	3.1 (2.4-4.2)	2.9 (2.2-3.6)	**3.4 (3-4.9)**[#]
DBP (mmHg)	73 (9.7)	74 (9.3)	72 (10.1)		ADMA* (µmol/l)	0.60 (0.53-0.65)	0.56 (0.52-0.61)	**0.62 (0.56-0.68)**[#]
PP (mmHg)	61 (13.4)	61(14.5)	62 (12.4)		SDMA* (µmol/l)	0.94 (0.74-1.30)	0.75 (0.61-0.91)	**1.27 (1.02-1.95)**[#]
Heart rate (1/min)	66 (12.3)	65 (11.0)	68 (13.5)		ACh* (%)	413 (244-614)	436 (276-610)	407 (202-691)
eGFR (ml/min/1.73m^2)	40.1 (21.7)	56.1 (18.9)	**23.8 (7.12)** [#]		SNP* (%)	454 (208-766)	602 (214-791)	372 (179-709)
Hemoglobin (g/l)	126 (14.4)	131 (15.6)	**122**[#] **(11.7)**		$PORH_{HA}$* (PU * sec)	592 (280-1047)	569 (266-1047)	690 (311-1069)

Continuous data are presented as mean (SD) or *median (interquartile range). [#] $p<0.05$. Categorical parameters are presented as % (n).

BMI: body mass index; Framingham score: Framingham 10 years cardiovascular risk score; SBP: systolic blood pressure; DBP: diastolic blood pressure; PP: pulse pressure; eGFR: estimated glomerular filtration rate; PTH: parathormone; CRP: C-reactive protein; ACR: urinary albumin to creatinine ratio; Ang-2: angiopoietin-2; ADMA: asymmetric dimethylarginin; SDMA: symmetric dimethylarginin. ACh: maximum percent laser Doppler flow increase in the forearm skin capillaries after acetylcholine iontophoresis; SNP: maximum percent laser Doppler flow increase in the forearm skin capillaries after sodium nitroprusside iontophoresis; $PORH_{HA}$: postocclusive reactive hyperemia area; PU: perfusion unit;

Table 2. Spearman correlation coefficients and corresponding p-values between the laser Doppler and biochemical microvascular parameters.

	ACh	SNP	$PORH_{HA}$	Ang-2	ADMA	SDMA
ACh	1 0.0	**0.498** **0.001**	**0.280** **0.010**	-0.161 0.145	-0.045 0.683	-0.112 0.310
SNP		1 0.0	**0.233** **0.033**	-0.170 0.122	-0.153 0.164	-0.038 0.734
$PORH_{HA}$			1 0.0	0.114 0.273	0.015 0.883	0.112 0.283
Ang-2				1 0.0	**0.292** **0.003**	**0.332** **0.001**
ADMA					1 0.0	**0.308** **0.002**
SDMA						1 0.0

ACh: maximum percent laser Doppler flow increase in the forearm skin capillaries after acetylcholine iontophoresis; SNP: maximum percent laser Doppler flow increase in the forearm skin capillaries after sodium nitroprusside iontophoresis; $PORH_{HA}$: postocclusive reactive hyperemia area; Ang-2: angiopoietin-2; ADMA: asymmetric dimethylarginin; SDMA: symmetric dimethylarginin.

Table 3. Variables associated with baseline laser Doppler and biochemical microvascular parameters.

	dependent variable: lnACh				**model R^2=0.106**
explanatory variable		estimate	SE	p	partial R^2
	diabetes	-0.598	0.192	0.003	0.106
	dependent variable: lnSNP				**model R^2=0.255**
explanatory variable		estimate	SE	p	partial R^2
	diabetes	-0.470	0.157	0.004	0.080
	antiplatelet	-0.435	0.152	0.005	0.069
	diuretic	-0.404	0.172	0.021	0.049
	dependent variable: lnPORH$_{HA}$				**model R^2=0.394**
explanatory variable		estimate	SE	p	partial R^2
	PP	-0.020	0.005	<0.001	0.119
	CCB	-0.600	0.127	<0.001	0.161
	fibrate	-0.391	0.180	0.032	0.033
	dependent variable: lnAng-2				**model R^2=0.415**
explanatory variable		estimate	SE	p	partial R^2
	cholesterol	-0.171	0.035	<0.001	0.136
	phosphorus	0.484	0.180	<0.001	0.112
	lnCRP	0.133	0.046	0.004	0.064
	heart rate	0.009	0.003	0.007	0.050
	age	0.006	0.003	0.041	0.027
	lnACR	0.048	0.020	0.047	0.026
	dependent variable: lnADMA				**model R^2=0.168**
explanatory variable		estimate	SE	p	partial R^2
	age	0.005	0.001	0.001	0.078
	heart rate	0.005	0.001	0.002	0.055
	phosphorus	0.157	0.079	0.049	0.034
	dependent variable: lnSDMA				**model R^2=0.760**

explanatory variable		estimate	SE	p	partial R^2
	eGFR	-0.013	0.001	<0.001	0.395
	phosphorus	0.352	0.107	0.001	0.034
	BMI	-0.012	0.005	0.009	0.020
	heart rate	0.005	0.002	0.002	0.023
	lnPTH	0.078	0.030	0.012	0.021
	lnACR	0.026	0.012	0.032	0.015
	RAASinh	-0.147	0.072	0.044	0.011

The variables considered in the multivariate linear regression models were age, sex, BMI, current smoking, diabetes, baseline cardiovascular disease, eGFR, lnCRP, hemoglobin, cholesterol, phosphorus, lnPTH, albumin, lnACR, systolic blood pressure, brachial pulse pressure, heart rate, use of ACE-inhibitor or ARB, diuretics, beta-blockers, alpha-blockers, calcium channel blockers, centrally effective antihypertensive drugs, nitrate, statin, fibrate, oral antidiabetic drugs, insulin, antiplatelet drugs.

Abbreviations: ln - natural logarithm; BMI: body mass index; eGFR: estimated glomerular filtration rate; PTH: parathormone; CRP: C-reactive protein; ACR: urinary albumin to creatinine ratio; ACh: maximum percent laser Doppler flow increase in the forearm skin capillaries after acetylcholine iontophoresis; SNP: maximum percent laser Doppler flow increase in the forearm skin capillaries after sodium nitroprusside iontophoresis; $PORH_{HA}$: post-occlusive reactive hyperemia area; ADMA: asymmetric dimethylarginin; SDMA: symmetric dimethylarginin; CCB: calcium-channel blocker; RAASinh: ACE-inhibitors or ARBs.

Table 4. Uni- and multivariate multiple failure time Cox-proportional hazards regression analyses of predictors of cardiovascular morbidity and mortality

	Outcome: CV mortality plus CV events				**Outcome: all-cause mortality plus CV events**			
	Univariate model				**Univariate model**			
	Hazard Ratio	**95% confidence interval**		**p**	**Hazard Ratio**	**95% confidence interval**		**p**
lnACh	0.79	0.59	1.05	0.112	0.86	0.64	1.15	0.319
lnSNP	0.93	0.59	1.48	0.789	1.02	0.67	1.56	0.893
lnPORH$_{HA}$	***0.69***	***0.57***	***0.84***	***<0.001***	***0.75***	***0.61***	***0.92***	***0.005***
lnAng-2	***1.99***	***1.25***	***3.19***	***0.004***	***2.02***	***1.31***	***3.13***	***<0.001***
lnADMA	1.03	0.23	4.56	0.96	1.59	0.41	6.12	0.49
lnSDMA	1.25	0.64	2.46	0.50	1.51	0.83	2.73	0.17
Age	***1.02***	***1.00***	***1.04***	***0.048***	***1.02***	***1.00***	***1.04***	***0.009***
Male gender	0.76	0.43	1.32	0.34	0.84	0.51	1.38	0.50
Current smoking	0.52	0.17	1.55	0.24	0.67	0.31	1.48	0.33
Diabetes	***3.23***	***1.71***	***6.08***	***<0.001***	***2.58***	***1.52***	***4.39***	***<0.001***
CV disease	***2.49***	***1.20***	***5.13***	***0.013***	***2.72***	***1.42***	***5.21***	***0.002***
BMI	1.05	0.99	1.11	0.054	1.03	0.98	1.08	0.18
eGFR	0.98	0.96	1.00	0.058	***0.98***	***0.96***	***0.99***	***0.015***
Hemoglobin	0.99	0.97	1.01	0.50	***0.98***	***0.96***	***0.99***	***0.019***
Cholesterol	0.85	0.68	1.07	0.18	0.84	0.67	1.04	0.12
Potassium	0.64	0.37	1.11	0.11	0.77	0.47	1.26	0.30
Calcium	1.95	0.13	30.2	0.63	1.65	0.16	17.2	0.68
Phosphorus	0.67	0.21	2.12	0.50	1.10	0.40	3.03	0.84
Albumin	1.03	0.95	1.11	0.38	1.01	0.95	1.07	0.69
lnCRP	1.32	0.98	1.79	0.067	***1.38***	***1.05***	***1.803***	***0.018***
lnPTH	1.20	0.86	1.68	0.26	1.11	0.81	1.51	0.50
lnACR	1.04	0.90	1.19	0.57	1.03	0.91	1.17	0.55
SBP	1.01	0.99	1.03	0.092	1.01	0.99	1.03	0.056
PP	***1.02***	***1.00***	***1.05***	***0.013***	***1.03***	***1.01***	***1.05***	***<0.001***
Heart rate	1.01	0.99	1.03	0.13	1.01	0.99	1.03	0.094
RAASinh	0.64	0.29	1.33	0.22	0.71	0.35	1.42	0.33
Diuretic	1.63	0.73	3.64	0.23	1.56	0.79	3.09	0.20
CCB	1.44	0.82	2.54	0.21	1.33	0.80	2.19	0.27
Beta-blocker	1.14	0.58	2.23	0.70	1.10	0.62	1.96	0.75
Alpha-blocker	1.63	0.93	2.86	0.090	1.48	0.88	2.49	0.14
Centrally eff.	1.38	0.64	2.95	0.41	1.22	0.59	2.51	0.59
Statin	0.89	0.51	1.54	0.67	0.71	0.44	1.16	0.17
Fibrate	***2.33***	***1.10***	***4.91***	***0.027***	***2.62***	***1.31***	***5.22***	***0.006***
Nitrate	1.17	0.61	2.23	0.64	0.91	0.49	1.68	0.77
OAD	***2.18***	***1.24***	***3.85***	***0.007***	***1.83***	***1.08***	***3.10***	***0.024***

Insulin	***2.04***	***1.08***	***3.87***	***0.028***	1.68	0.94	3.00	0.08
Antiplatelet	1.20	0.64	2.27	0.57	1.29	0.74	2.25	0.37
	Final multivariate model				**Final multivariate model**			
	Hazard Ratio	**CI 95%**		**p**	**Hazard Ratio**	**CI 95%**		**p**
ln $PORH_{HA}$	***0.71***	***0.57***	***0.89***	***0.003***	**-**	**-**	**-**	**-**
Age	***1.04***	***1.01***	***1.06***	***0.004***	***1.03***	***1.00***	***1.06***	***0.011***
lnCRP	***1.26***	***1.00***	***1.57***	***0.045***	**-**	**-**	**-**	**-**
Diabetes	***4.81***	***2.13***	***10.88***	***<0.001***	***2.74***	***1.56***	***4.82***	***<0.001***
PP	**-**	**-**	**-**	**-**	***1.03***	***1.01***	***1.05***	***0.005***
Fibrate	**-**	**-**	**-**	**-**	***2.27***	***1.24***	***4.17***	***0.008***

Hazard ratios represent a unit increase in the variable.

Abbreviations: CV: cardiovascular; ln: natural logarithm; ACh: maximum percent laser Doppler flow increase in the forearm skin capillaries after acetylcholine iontophoresis; SNP: maximum percent laser Doppler flow increase in the forearm skin capillaries after sodium nitroprusside iontophoresis; $PORH_{AH}$: post-occlusive reactive hyperemia area; Ang-2: angiopoietin-2; ADMA: asymmetric dimethylarginin; SDMA: symmetric dimethylarginin; BMI: body mass index; eGFR: estimated glomerular filtration rate; CRP: C-reactive protein; PTH: parathormone; ACR: urinary albumin-to-creatinine ratio; SBP: peripheral systolic blood pressure; PP: pulse pressure; RAASinh: ACE-inhibitors plus ARBs; CCB: calcium channel blockers; Centrally eff: centrally effective antihypertensive drugs; OAD: oral antidiabetic drugs; Antiplatelet: antiplatelet drugs.