eNOS and Caveolin-1 Gene Polymorphisms Interaction and Intima Media Thickness: A Proof of Concept Study in ESRD Patients

Alessandra Testa[1], Belinda Spoto[1], Maria Cristina Sanguedolce[1], Rosa M. Parlongo[1], Anna Pisano[1], Giovanni Tripepi[1], Francesco A. Benedetto[2], Francesca Mallamaci[1] and Carmine Zoccali[1]

BACKGROUND

Caveolae are a prominent microdomain in endothelial cells and appropriate localization in caveolae is fundamental for endothelial nitric oxide synthase (eNOS) activity. Since the Glu298Asp variant in the *eNOS* gene alters caveolar localization of the corresponding enzyme, we tested the interaction between this variant and the rs4730751 polymorphism of the caveolin-1 (*CAV-1*) gene as related to arterial remodeling in end-stage renal disease (ESRD) patients.

METHODS

One hundred and thirty-three ethnically homogeneous ESRD patients underwent carotid ultrasonographic studies to measure intima-media thickness (IMT) and carotid cross-sectional area (CSA). Genotyping was performed by high-throughput allelic discrimination assays on real-time PCR.

RESULTS

Arterial remodeling was associated to the number of G alleles of *CAV-1* polymorphism, GG homozygotes displaying an IMT and a CSA that were, respectively, 16% and 21% higher than those in patients without the risk allele ($P < 0.012$). In multiple linear regression analyses including the *CAV-1* and the *eNOS* polymorphisms and adjusting for classical risk factors and risk factors peculiar to ESRD both polymorphisms were independent correlates of IMT (*CAV-1*: $\beta = 0.20$, $P = 0.01$; *eNOS* $\beta = 0.25$, $P = 0.001$) and CSA (*CAV-1*: $\beta = 0.20$, $P = 0.01$: *eNOS* $\beta = 0.13$, $P = 0.09$). Furthermore, strong interactions emerged between the two polymorphisms for explaining the variability in IMT ($P = 0.001$) and in CSA ($P = 0.038$) in these patients.

CONCLUSION

Overall these findings form preliminary evidence that disturbed interaction between CAV-1 and eNOS may be of relevance for arterial disease in ESRD and perhaps in other human diseases.

Keywords: *blood pressure; cardiovascular risk; caveolin-1; end-stage renal disease; endothelial nitric oxide synthase; genetics; hypertension*

American Journal of Hypertension, advance online publication 6 October 2011; doi:10.1038/ajh.2011.178

Severe arterial disease is a major feature of end-stage renal disease (ESRD) and coherent lines of evidence implicate disturbed regulation of the nitric oxide (NO) system in cardiovascular damage in this condition.[1] Both, environmental risk factors like accumulation of the endogenous inhibitor of NO synthase (NOS), asymmetric dimethyl arginine (ADMA),[2] and genetic factors such as gene variants in the endothelial NOS (*eNOS*) gene concur in determining the severity of this disorder in this population.[3,4] As to the genetic component, we reported that the Glu298Asp *eNOS* polymorphism (rs1799983) is associated with intima-media thickness (IMT)[3] and wall to lumen ratio and that this gene variant interacts with ADMA to explain the variability in arterial remodeling in ESRD.[4]

Caveolae are a prominent microdomain in endothelial cells wherein fundamental cell signaling mechanisms are generated and/or transduced and it is precisely at this site that NOS (eNOS) achieves maximal activity.[5] Caveolin-1 (CAV-1) is a main structural component of this microdomain which is characterized by a strong biological interaction with eNOS. CAV-1 null aortas show a marked increase in acetylcholine induced vasorelaxation even at extremely low concentrations of acetylcholine, indicating that CAV-1 serves to constitutively inhibit eNOS activity.[6] Within caveolae, CAV-1 is in close proximity to cell membrane receptors for fibrotic mediators such as transforming growth factor-β,[7] a factor strongly implicated in atherosclerosis.[8] Furthermore, the *in vitro* observation that the *eNOS* gene variant (Glu298Asp), which we associated to arterial disease in ESRD, also alters caveolar localization of the *eNOS* gene and its functional link with CAV-1[9] highlights the potential relevance of the CAV-1–eNOS interaction in arterial disease in these patients. In this regard, it is of outmost interest that the rs4730751 polymorphism in *CAV-1* gene predicts allograft loss in ESRD patients submitted to

[1]CNR-IBIM, National Research Council-Institute of Biomedicine, Clinical Epidemiology and Physiopathology of Renal Diseases and Hypertension, Reggio Calabria, Italy; [2]Division of Cardiology, Morelli Hospital, Reggio Calabria, Italy. Correspondence: Carmine Zoccali (carmine.zoccali@tin.it)

Received 12 May 2011; first decision 21 July 2011; accepted 7 August 2011.

renal transplantation,[10] a phenomenon attributable to vascular proliferation and fibrosis triggered by this SNP. Because this polymorphism appears to be a most likely player in arterial disease, as a proof of the relevance of the CAV-1–eNOS interaction in man, we tested the relationship between the *CAV-1* polymorphism and arterial remodeling in ESRD patients and analyzed the genetic interaction between this SNP and the Glu298Asp *eNOS* variant in the same set of patients where we originally described the association between the Glu298Asp genotype and arterial disease. To account for the relatively low sample size, the study results were validated by a bootstrap resampling technique whereby the reproducibility of the relationships between *CAV-1/eNOS* and the severity of carotid atherosclerosis was investigated in 1,000 random samples extracted from the original study population.

METHODS

The protocol conformed to the local ethical guidelines of our institution and was approved by the ethical committee of the "Azienda Ospedaliera Bianchi Melacrino Morelli." Informed consent was obtained from each participant.

Patients. One hundred thirty-three (79 men and 54 women, all Caucasians) dialysis patients (87 on hemodialysis and 46 on chronic ambulatory peritoneal dialysis) who had been on regular dialysis treatment for at least 6 months (duration of regular dialysis treatment: median 47 months, interquartile range: 18–115 months) and who were free of overt infections (fever, infected vascular access or peritonitis, or exit site infection) were recruited for the study. Hemodialysis patients were being treated three times weekly with standard bicarbonate dialysis (in mmol/l: Na 138, HCO_3 5, K 1.5, Ca 1.25, Mg 0.75) either with Cuprophan or semisynthetic membranes. The average urea fractional urea clearance (Kt/V) in these patients was 1.29 ± 0.29. The remaining 46 patients were on chronic ambulatory peritoneal dialysis (weekly Kt/V 1.66 ± 0.33). All hemodialysis patients were virtually anuric (24-h urine volume <200 ml/day), whereas a minority of chronic ambulatory peritoneal dialysis patients ($n = 6$) had a 24-h diuresis >200 ml/day. Nineteen patients were diabetics and 66 were habitual smokers (22 ± 18 cigarettes/day). Seventy-seven patients were on antihypertensive treatment (51 on monotherapy with angiotensin-converting enzyme inhibitors, angiotensin-1 antagonists, calcium-channel blockers, α- and β-blockers and the remaining 26 on double or triple therapy with various combinations of these drugs). Sixty-six patients were on treatment with erythropoietin.

Carotid ultrasonography. In all patients, ultrasonographic studies on common carotid arteries (CCA) were performed bilaterally by a single observer (F.A.B.) who was blinded as to the clinical and biochemical data.[11] All studies were performed with a Hewlett Packard Sonos 1500 (Palo Alto, CA) using a 7.5-MHz high resolution probe. IMT was defined as a low-level echo gray band that does not project into the arterial lumen and was measured during end-diastole as the distance from the leading edge of the second echogenic line of the far walls of the distal segment of the CCA, the carotid bifurcation, and the initial tract of internal carotid artery on both sides. Measurements were performed 0.5, 1, and 2 cm below and above the bifurcation (six measurements on each side) and the average measurement was taken as the IMT.[11] The cross-sectional area (CSA) of the CCA was measured bilaterally 2 cm below the bifurcation during end-diastole.[12] The number of atherosclerotic plaques (either as faint gray echoes (soft plaques) or bright white echoes (calcified plaque) protruding into the lumen)[13] detected in the bulbar area (from 2 cm below to 2 cm above the bifurcation) of the carotid arteries was recorded on both sides and summed up. The IMT and internal diameter of the CCA measurements were always performed in plaque-free arterial segments. Repeated studies in 105 dialysis patients by a blinded observer in our laboratory showed that the IMT and the internal diameter of the CCA represent reliable measurements because their coefficient of variation was 5.5 and 3.2%, respectively. In our laboratory abnormal IMT is defined as IMT >0.82 mm. This value corresponds to the average value + 2 s.d. in a group of 70 healthy Italian normotensive volunteers (age 52 ± 14 years).[14]

Laboratory measurements. For hemodialysis patients fasting blood sampling was performed during the midweek nondialysis day and for chronic ambulatory peritoneal dialysis patients at empty abdomen. Serum cholesterol, albumin, calcium and phosphate, and hemoglobin measurements were made using standard methods in the routine clinical laboratory. The C-reactive protein was measured by using a commercially available kit (Behring, Scoppito, L' Aquila, Italy). Plasma homocysteine and ADMA were determined as previously reported.[2]

Genotyping of the Cav-1 and eNOS gene polymorphisms. Genomic DNA was extracted from peripheral blood leukocytes by salting-out technique.[15] The participants were genotyped for a common single-nucleotide polymorphism (*G-894-T)* on exon 7 of *eNOS* gene on chromosome 7. This polymorphism, described under identification number rs1799983, was studied by the validated TaqMan SNP Genotyping Assay C_3219460_20. Genotyping of the *Cav-1* rs4730751 polymorphism on intron 1 of the gene on chromosome 7 was determined by the validated TaqMan SNP Genotyping Assay C_2972987_10. SNPs genotyping was performed by ABI PRISM 7900HT according to the manufacturer's recommendations (Applied Biosystems, Foster City, CA). The assays mix (including unlabeled PCR primers, FAM, and VIC dye-labeled TaqMan MGB probes) was designed and provided by Applied Biosystems. The reaction system contained 1–5 ng of genomic DNA, 12.5 µl of TaqMan Universal PCR Master Mix, 2× no AmpErase UNG, 1,25 µl 40 × assay mix and was adjusted with H_2O for a total volume of 25 µl. Alleles were scored using the allelic discrimination software Sequence Detection System v2.2 (Applied Biosystems). A random 5% of samples were independently repeated to confirm genotyping results. The genotype results for these samples were completely consistent.

Table 1 | Main clinical an biochemical characteristics of the study population

	Caveolin polymorphism			
	TT ($n = 16$)	GT ($n = 55$)	GG ($n = 62$)	*P* for trend
Age (years)	61 ± 17	60 ± 15	60 ± 16	0.93
Male sex, *n* (%)	9 (56%)	30 (55%)	40 (65%)	0.34
Diabetics, *n* (%)	1 (6.3%)	10 (18.2%)	8 (12.9%)	0.88
Smokers, *n* (%)	5 (31.3%)	25 (45.5%)	36 (58.1%)	0.04
On antihypertensive therapy *n* (%)	7 (44%)	36 (66%)	34 (55%)	0.93
On treatment with EPO, *n* (%)	9 (46.3%)	28 (51.0%)	29 (47.0%)	0.48
With previous CV events, *n* (%)	6 (37.5%)	31 (56.4%)	30 (48.4%)	0.83
Systolic pressure (mm Hg)	134 ± 17	137 ± 21	136 ± 21	0.76
Diastolic pressure (mm Hg)	73 ± 10	76 ± 12	76 ± 12	0.50
Heart rate (beats/min)	85 ± 9	84 ± 9	82 ± 16	0.34
Hemoglobin (g/l)	96 ± 22	102 ± 19	108 ± 20	0.02
Albumin (g/l)	40 ± 6	38 ± 6	38 ± 6	0.47
Calcium × phosphate ($mmol^2/l^2$)	4.6 ± 1.5	4.4 ± 1.3	4.4 ± 1.4	0.63
Cholesterol (mg/dl)	218 ± 55	209 ± 48	212 ± 60	0.86
ADMA (μmol/l)	4.6 (3.4–5.1)	4.0 (3.2–5.1)	4.0 (3.1–5.1)	0.44
CRP (mg/l)	6.4 (3.8–13.1)	7.4 (3.4–17.9)	9.2 (3.5–26.4)	0.28
Homocysteine (μmol/l)	20.5 (14.6–27.8)	26.4 (20.2–49.0)	32.2 (22.3–42.6)	0.07

ADMA, asymmetric dimethyl arginine; CRP, C-reactive protein; CV, cardiovascular; EPO, erythropoietin.

Statistical analysis. Data are expressed as mean ± s.d. or s.e., median (interquartile range), or as percent frequency, as appropriate. Groups comparisons were made by *P* for trend. The relationship between *CAV-1-eNOS* genotypes (defined according to a codominant model) and the severity of carotid atherosclerosis (as estimated by IMT, CSA and total number of atherosclerotic plaques) was analyzed by univariate and multivariate regression analyses. For multivariate modeling, we consider the following variables: *CAV-1* and *eNOS* genotypes as well as a series of traditional risk factors (age, sex, smoking, cardiovascular comorbidities, diabetes, cholesterol, systolic pressure, and antihypertensive treatment), factors peculiar to ESRD (hemoglobin, albumin, calcium phosphate product) and emerging risk factors (C-reactive protein, homocysteine, and ADMA). The interaction analysis between caveolin and *eNOS* genes for explaining the severity of atherosclerosis was analyzed by adding into the multivariate models the *CAV-1*–*eNOS* polymorphisms interaction term. Into the final models, we included all variables that were related (with $P \leq 0.15$) to the exposure (*CAV-1* and *eNOS* genotypes and their interaction term) or to the study outcomes (IMT and CSA). By this strategy, we constructed models of adequate statistical power (at least 10 patients for each variable into the models). The estimated increase in the severity of carotid atherosclerosis associated to 1 G allele increase of the *CAV-1* gene was calculated by linearly combining the regression coefficients (see Appendix for mathematical details). To validate the independent relationships of *CAV-1*, *eNOS* and their interaction term with indicators of carotid atherosclerosis (IMT and CSA) a bootstrap resampling technique of 1,000 samples (extracted randomly from the original sample) was performed.[16] Data are expressed as regression coefficients (b), standardized regression coefficients (β) and *P* values. All calculations were done by a standard statistical package (STATA for Windows version 9.0, College Station, TX).

RESULTS

Both *CAV-1* (TT: 12%; GT: 41%; GG: 47%) and *eNOS* (TT: 12%; GT: 43%; GG: 45%) genotypic distributions did not deviate from Hardy–Weinberg equilibrium ($\chi^2 = 0.49$, $P = 0.48$ and $\chi^2 = 1.60$, $P = 0.20$, respectively). The demographic and clinical characteristics of patients stratified according to *CAV-1* polymorphism are presented in **Table 1**. GG homozygotes for this polymorphism had higher hemoglobin and were more frequently smokers as compared to those with other genotypes. No differences were observed as for the remaining demographic, clinical or biochemical data. A descriptive analysis according to *eNOS* polymorphism was reported elsewhere.[3]

CAV-1 polymorphism and carotid ultrasonography

IMT (1.04 ± 0.24 mm) was above the upper limit of the corresponding normal range in 114 (86%) ESRD patients. CSA was on average 12.2 ± 3.5 mm^2. On univariate analysis, the number of G alleles of *CAV-1* polymorphism was unrelated to the total number of atherosclerotic plaques (*P* = NS).

On the other hand, IMT and CSA increased according to the number of risk alleles (G allele) of *CAV-1* polymorphism (**Figure 1**), so that patients with the GG genotype had an IMT and a CSA that were 16% and 21% higher than those of patients without the risk allele, respectively (**Figure 1**). The

number of T alleles of the *eNOS* polymorphism was strongly related with IMT ($P = 0.005$) and tended to correlate with CSA ($P = 0.14$). In multiple linear regression models including the *CAV-1* and the *eNOS* polymorphisms and adjusting for all risk factors listed in Table 1, both polymorphisms resulted to be independent correlates of IMT (*CAV-1*: $\beta = 0.20$, $P = 0.01$; *eNOS* $\beta = 0.25$, $P = 0.001$)) and CSA (*CAV-1*: $\beta = 0.20$, $P = 0.01$: *eNOS* $\beta = 0.13$, $P = 0.09$) and bootstrapping validation showed that these links were highly consistent (IMT-*CAV-1*: $\beta = 0.20$; IMT-*eNOS* $\beta = 0.24$; CSA-*CAV-1*: $\beta = 0.19$: CSA-*eNOS* $\beta = 0.13$).

CAV-1, *eNOS* polymorphisms, and carotid ultrasonography: interaction analysis

On crude analysis, *eNOS* polymorphism modified the relationship between the number of *CAV-1* risk alleles and IMT. Indeed, the presence of the risk allele of the *CAV-1* gene was associated with a more marked increase in IMT in patients with the TT *eNOS* genotype as compared to that observed in patients with TG and GG genotypes (P for interaction = 0.018) (Table 2 and Figure 2). The interaction between the two polymorphisms for explaining the IMT was even stronger ($P = 0.001$) after data adjustment for traditional and nontraditional risk factors (Table 2, last column) and remained meaningful also after bootstrapping validation (Figure 2). On crude analysis, *eNOS* polymorphism modifies the *CAV-1*–CSA link (Figure 2) but this effect was not statistically significant ($P = 0.23$). However, after data adjustment for all risk factors listed in Table 2, an interaction between the two polymorphisms emerged for explaining the variability in CSA ($P = 0.038$). The estimated change in CSA for each increase in T allele number was $2.6 \pm 0.5\,mm^2$ in TT, $1.5 \pm 0.5\,mm^2$ in TG, and $0.4 \pm 0.5\,mm^2$ in GG *eNOS* genotype patients (P for interaction = 0.038) (Figure 2) and again this relationship maintained after bootstrapping validation (Figure 2).

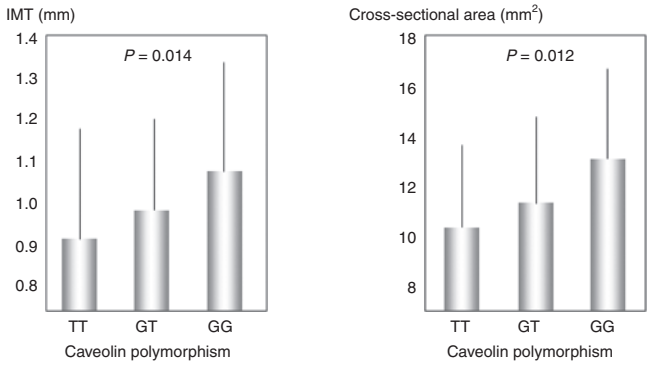

Figure 1 | Relationships between genotypes of caveolin-1 polymorphism (codominant model) and intima-media thickness and cross-sectional area. Data are mean and s.d. Comparisons were made by *P* for trend. IMT, intima-media thickness.

DISCUSSION

In this proof-of-concept study in patients with ESRD, we associate for the first time a polymorphism in the *CAV-1* gene with intimal thickening and arterial dilatation and show a coherent interaction of the same polymorphism with an established genetic marker of arterial disease in ESRD, i.e., the Glu298Asp variant in the *eNOS* gene.

From a pathology point of view, arterial disease in ESRD is primarily a medial degenerative condition spread from the thoracic aorta and central arteries to peripheral arteries, causing dilation, diffuse hypertrophy, and stiffening.[17] Accordingly, intimal thickening and outward remodeling of arterial vessels, as measured by IMT and CSA in echo-color Doppler studies of carotid arteries, are fundamental characteristics of arterial involvement in ESRD patients.[17] As opposed to plaque formation which is an inflammation-driven process in ESRD,[13] arterial remodeling is a

Table 2 | Multiple linear regression model of intima-media thickness

		Crude			Fully adjusted model		
	Units of increase	TT *eNOS* genotype	TG *eNOS* genotype	GG *eNOS* genotype	TT *eNOS* genotype	TG *eNOS* genotype	GG *eNOS* genotype
Caveolin polymorphism	1 G allele	0.22 ± 0.04	0.11 ± 0.04	0.01 ± 0.04	0.24 ± 0.04	0.12 ± 0.04	0.003 ± 0.04
		P for interaction = 0.018			*P* for interaction = 0.001		
Age	1 year				0.005 ± 0.001, *P* < 0.001		
Gender	0 = F; 1 = M				0.02 ± 0.045, *P* = 0.66		
Smoking	0 = no; 1 = yes				−0.004 ± 0.045, *P* = 0.86		
Systolic pressure	1 mm Hg				0.0013 ± 0.001, *P* = 0.12		
CV comorbidities	0 = no; 1 = yes				0.035 ± 0.04, *P* = 0.33		
Hemoglobin	1 g/dl				−0.02 ± 0.001, *P* = 0.01		
Albumin	1 g/dl				0.001 ± 0.003, *P* = 0.81		
Homocysteine	1 log µmol/l				0.17 ± 0.07, *P* = 0.02		
ADMA	1 log µmol/l				0.28 ± 0.11, *P* = 0.01		
CRP	1 log mg/l				0.07 ± 0.04, *P* = 0.11		

Data are regression coefficients ± s.e. and *P* values.
ADMA, asymmetric dimethyl arginine; CRP, C-reactive protein; CV, cardiovascular; eNOS, endothelial nitric oxide synthase.

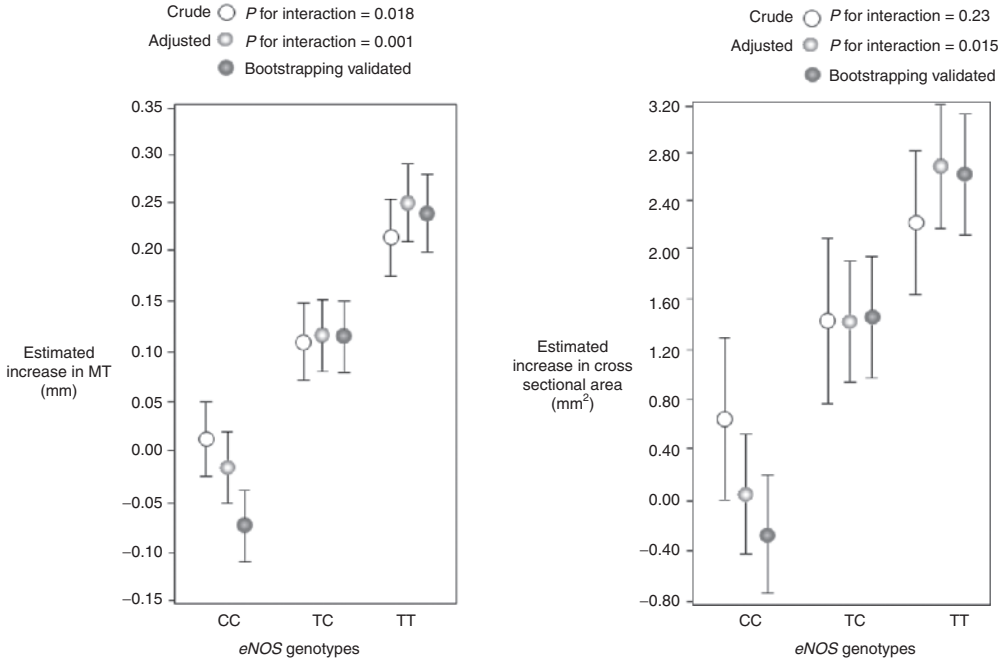

Figure 2 | Effect modification of endothelial nitric oxide synthase (*eNOS*) genotypes on the crude, adjusted, and bootstrapping validated increase in intima-media thickness (IMT) and cross-sectional area associated to 1 G allele increase of caveolin-1 polymorphism. Data are mean ± s.e.

phenomenon mainly determined by reduced NO bioavailability in these patients. Indeed intimal thickening is strongly associated both with plasma ADMA levels and with the Glu298Asp polymorphism,[3] a gene variant which alters eNOS activity. The fundamental role of reduced NO bioavailability in vascular damage in ESRD is also highlighted by the fact that both ADMA and the Glu298Asp variant are also strong predictors of incident cardiovascular complications in these patients.[4]

The plasma membrane invaginations that form caveolae are a critical site for modulation of eNOS activity. Indeed it is within caveolae that eNOS attains maximal activity and interacts with CAV-1, a 21–24 kDa protein that coats the cytoplasmic surface of caveolae. In caveolae, eNOS activation is modulated through direct-steric inhibition of calmodulin binding with caveolin.[18] CAV-1 null aortas show a marked increase in acetylcholine-induced vasorelaxation even at extremely low concentrations of acetylcholine, indicating that CAV-1 serves to constitutively inhibit eNOS activity.[6] Thus dissociation from its inhibitory interaction with CAV-1 is a fundamental step for the activation of eNOS. Agonist activation (or stimulation by shear) increases intracellular calcium and calcium-calmodulin binding, which displaces caveolin and reverses its inhibitory effect on eNOS. In addition to this tonic inhibition, interaction with CAV-1 contributes to eNOS concentration in caveolae. As previously alluded to, although a substantial proportion of active eNOS resides in the peri-Golgi area, proper caveolar localization is critical for eNOS activation and maximal activity.[19]

The Glu298Asp variant is one of the most coherent genetic marker of high risk for coronary artery disease and myocardial infarction[20] and hypertension[21] in various populations and for arterial remodeling[3] and incident cardiovascular events[4] in ESRD. It was shown that this variant disturbs the catalytic activity of eNOS but the precise biological alteration underlying the high risk of this gene variant is still debated. Given the high risk of the Glu298Asp variant and the importance of the interaction of eNOS with CAV-1 for the location of this enzyme in caveolae and the control of its activity, the Glu298Asp variant is suspected to be a critical factor in the CAV-1–eNOS interaction.[9] Indeed this gene variant decreases the degree of interaction of eNOS with Cav-1, hinders location of eNOS in caveolae and diminishes shear-dependent NOS activation.[9] On the other hand, the recent discovery that a polymorphism (rs4730751) in *CAV-1* gene targeting vascular proliferation and fibrosis coherently predicted graft failure in two separate cohorts of ESRD patients followed-up for over 10 years after renal transplantation[10] suggests that this variant can modify the interaction between the corresponding gene product, CAV-1, and other proteins, particularly eNOS, an enzyme that is maximally activated only when localized in caveolae. In line with this hypothesis, we found that ESRD patients homozygous for the risk allele of the rs4730751 polymorphism exhibited intima media thickening (i.e., vascular hypertrophy) as well as outward arterial remodeling (enlargement in CSA). Furthermore, the presence of the risk allele for the *CAV-1* polymorphism determined a tripling in the dose-dependent increase in both IMT and CSA in individuals homozygous for the risk allele of the *eNOS* Glu298Asp variant as compared to those with other *eNOS* genotypes pointing to a relevant interaction between these two gene variants. Overall these findings form a preliminary hypothesis generating evidence that disturbed interaction between CAV-1 and eNOS may be of relevance for arterial disease in ESRD and perhaps in other human diseases.

Our study is limited in many respects. The most obvious limitation is sample size. Studies reporting false positive associations

of gene variants and clinical end-points abound and large sample size and confirmatory analyses in diverse populations are recommended for testing genetic associations. In this regard, it is important noting that a bootstrapping validation, simulating a re-sampling of 1,000 samples randomly extracted from the original population, showed that the link between *CAV-1* and caveolin–eNOS interaction term with the severity of carotid atherosclerosis was highly consistent. In the background knowledge linking *eNOS* gene and arterial remodeling in ESRD, the present study should be seen as a proof of concept analysis specifically stimulated by the recent identification of a *CAV-1* gene variant associated with vascular proliferation, fibrosis, and organ (kidney) failure. The hypothesis we probed had a precise biological rationale based on the experimental *in vitro* interaction between the *Glu298Asp* gene variant with *CAV-1*. Confirmatory observations in other ESRD cohorts and mechanistic studies are needed to clarify the effect modification by the *Glu298Asp* gene variant to the link between the rs4730751 polymorphism of *CAV-1* gene and arterial remodeling in this population.

APPENDIX

Calculation of the estimated slope in IMT associated to *CAV-1* polymorphism (1 G allele increase) across the number of T allele (0, 1, and 2) of the *eNOS* genotype.

Multiple regression analysis of Caveolin 1 × *eNOS* interaction term for predicting intima-media thickness (see table below).

	Regression coefficients
Caveolin 1 × *eNOS* interaction term	0.121*
Caveolin-1	0.00279
eNOS	−0.0835
Age	0.005
Gender	0.02
Smoking	−0.004
Systolic pressure	0.0013
CV comorbidities	0.035
Hemoglobin	−0.02
Albumin	0.001
Homocysteine	0.17
ADMA	0.28
CRP	0.07

*$P = 0.001$.
CRP, C-reactive protein; CV, cardiovascular.

A significant interaction between the two polymorphisms implies that the slope in IMT associated to Caveolin-1 polymorphism (1 G allele increase) must be calculated at prespecified values of the number of T alleles of the *eNOS* genotype by linearly combining the corresponding regression coefficients derived from multiple regression analysis. Then, the estimated slope in IMT associated to 1 G allele increase at 0, 1, and 2 T alleles of the *eNOS* genotype are calculated as follows:

- Slope in IMT associate 1 G allele increase$_{(eNOS\ =\ 0)}$ = $0.00279 + 0.121 \times 0 = 0.003$
- Slope in IMT associate 1 G allele increase$_{(eNOS\ =\ 1)}$ = $0.00279 + 0.121 \times 1 = 0.12$
- Slope in IMT associate 1 G allele increase$_{(eNOS\ =\ 2)}$ = $0.00279 + 0.121 \times 2 = 0.24$

Disclosure: The authors declared no conflict of interest.

1. Zoccali C. Asymmetric dimethylarginine (ADMA): a cardiovascular and renal risk factor on the move. *J Hypertens* 2006; 24:611–619.
2. Zoccali C, Bode-Böger S, Mallamaci F, Benedetto F, Tripepi G, Malatino L, Cataliotti A, Bellanuova I, Fermo I, Frölich J, Böger R. Plasma concentration of asymmetrical dimethylarginine and mortality in patients with end-stage renal disease: a prospective study. *Lancet* 2001; 358:2113–2117.
3. Belinda S, Francesco AB, Alessandra T, Giovanni T, Francesca M, Renke M, Boeger RH, Zoccali C. Atherosclerosis and the Glu298Asp polymorphism of the eNOS gene in white patients with end-stage renal disease. *Am J Hypertens* 2005; 18:1549–1555.
4. Testa A, Spoto B, Tripepi G, Mallamaci F, Malatino L, Fatuzzo P, Maas R, Boeger R, Zoccali C. The GLU298ASP variant of nitric oxide synthase interacts with asymmetric dimethyl arginine in determining cardiovascular mortality in patients with end-stage renal disease. *J Hypertens* 2005; 23:1825–1830.
5. Razani B, Lisanti MP. Caveolin-deficient mice: insights into caveolar function human disease. *J Clin Invest* 2001; 108:1553–1561.
6. Razani B, Engelman JA, Wang XB, Schubert W, Zhang XL, Marks CB, Macaluso F, Russell RG, Li M, Pestell RG, Di Vizio D, Hou H Jr, Kneitz B, Lagaud G, Christ GJ, Edelmann W, Lisanti MP. Caveolin-1 null mice are viable but show evidence of hyperproliferative and vascular abnormalities. *J Biol Chem* 2001; 276:38121–38138.
7. Santibanez JF, Blanco FJ, Garrido-Martin EM, Sanz-Rodriguez F, del Pozo MA, Bernabeu C. Caveolin-1 interacts and cooperates with the transforming growth factor-beta type I receptor ALK1 in endothelial caveolae. *Cardiovasc Res* 2008; 77:791–799.
8. Grainger DJ. TGF-beta and atherosclerosis in man. *Cardiovasc Res* 2007; 74:213–222.
9. Joshi MS, Mineo C, Shaul PW, Bauer JA. Biochemical consequences of the NOS3 Glu298Asp variation in human endothelium: altered caveolar localization and impaired response to shear. *FASEB J* 2007; 21:2655–2663.
10. Moore J, McKnight AJ, Simmonds MJ, Courtney AE, Hanvesakul R, Brand OJ, Briggs D, Ball S, Cockwell P, Patterson CC, Maxwell AP, Gough SC, Borrows R. Association of caveolin-1 gene polymorphism with kidney transplant fibrosis and allograft failure. *JAMA* 2010; 303:1282–1287.
11. Benedetto FA, Mallamaci F, Tripepi G, Zoccali C. Prognostic value of ultrasonographic measurement of carotid intima media thickness in dialysis patients. *J Am Soc Nephrol* 2001; 12:2458–2464.
12. Khattar RS, Acharya DU, Kinsey C, Senior R, Lahiri A. Longitudinal association of ambulatory pulse pressure with left ventricular mass and vascular hypertrophy in essential hypertension. *J Hypertens* 1997; 15:737–743.
13. Benedetto FA, Tripepi G, Mallamaci F, Zoccali C. Rate of atherosclerotic plaque formation predicts cardiovascular events in ESRD. *J Am Soc Nephrol* 2008; 19:757–763.
14. Rossi A, Baldo-Enzi G, Calabrò A, Sacchetto A, Pessina AC, Rossi GP. The renin-angiotensin-aldosterone system and carotid artery disease in mild-to-moderate primary hypertension. *J Hypertens* 2000; 18:1401–1409.
15. Miller SA, Dykes DD, Polesky HF. Salting out procedure for extracting DNA from human nucleated cells. *Nucl Acids Res* 1988; 16:1215.
16. Steverberg EW. *Clinical Prediction Models. A Practical Approach to Development, Validation, and Updating*. Springer: New York, 2009. pp. 93–98 and 303–304.
17. London GM, Drueke TB. Atherosclerosis and arteriosclerosis in chronic renal failure. *Kidney Int* 1997; 51:1678–1695.
18. Michel JB, Feron O, Sacks D, Michel T. Reciprocal regulation of endothelial nitric-oxide synthase by Ca2+-calmodulin and caveolin. *J Biol Chem* 1997; 272:15583–15586.
19. Rahman A, Swärd K. The role of caveolin-1 in cardiovascular regulation. *Acta Physiol (Oxf)* 2009; 195:231–245.
20. Casas JP, Bautista LE, Humphries SE, Hingorani AD. Endothelial nitric oxide synthase genotype and ischemic heart disease: meta-analysis of 26 studies involving 23028 subjects. *Circulation* 2004; 109:1359–1365.
21. Hingorani AD. Endothelial nitric oxide synthase polymorphisms and hypertension. *Curr Hypertens Rep* 2003; 5:19–25.