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Association of plasma asymmetrical dimethylarginine (ADMA) with elevated vascular superoxide production and endothelial nitric oxide synthase uncoupling: implications for endothelial function in human atherosclerosis

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Background Asymmetrical dimethylarginine (ADMA), an endogenous inhibitor of endothelial nitric oxide synthase (eNOS), is considered to be a risk factor for atherosclerosis. However, the mechanisms relating ADMA with vascular function have been evaluated *in vitro* and in animal models, but its effect in human vasculature is unclear.

Aims We examined the impact of serum ADMA on endothelial nitric oxide (NO) bioavailability and vascular superoxide radical (O2-) production in patients with advanced atherosclerosis.

Methods and results Paired samples of saphenous veins (SVs) and internal mammary arteries (IMAs) were collected from 201 patients undergoing coronary bypass surgery, and serum ADMA was measured pre-operatively. The vasomotor responses of SV segments to acetylcholine (ACh) and bradykinin (Bk) were evaluated *ex vivo*. Vascular O2- was measured in paired SV and IMA by lucigenin-enhanced chemiluminescence. The L-NAME-inhibitable as well as the NADPH-stimulated vascular O2- generation was also determined by chemiluminescence. High serum ADMA levels were associated with decreased vasorelaxation of SV to ACh ($P < 0.05$) and Bk ($P < 0.05$). Similarly, high serum ADMA was associated with higher total O2- production in both SVs and IMAs ($P < 0.05$) and greater L-NAME-inhibitable vascular O2- ($P < 0.05$). However, serum ADMA was not associated with NADPH-stimulated vascular O2-. In multivariable linear regression, serum ADMA was independently associated with vascular O2- in both SVs [β (SE): 0.987 (0.412), $P = 0.019$] and IMAs [β (SE): 1.905 (0.541), $P = 0.001$]. Asymmetrical dimethylarginine was also independently associated with maximum vasorelaxation in response to both ACh [β (SE): 14.252 (3.976), $P = 0.001$] and Bk [β (SE): 9.564 (3.762), $P = 0.013$].

Conclusion This is the first study that demonstrates an association between ADMA and important measures of vascular function, such as vascular O2- production and NO bioavailability directly in human vessels. Although serum ADMA has no effect on NADPH-stimulated superoxide in intact vessels, it is associated with greater eNOS uncoupling in the human vascular endothelium of patients with coronary artery disease.

Keywords Endothelial nitric oxide synthase • Asymmetrical dimethylarginine • Nitric oxide • Superoxide

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Introduction

Vascular oxidative stress is a major factor in the pathogenesis of atherosclerosis.[1] Reactive oxygen species lead to endothelial dysfunction, induce oxidation of low-density lipoprotein leading to the formation of oxidized low-density lipoprotein (ox-LDL), and activate redox-sensitive proinflammatory signalling pathways.[2] Superoxide is produced in the vascular wall by enzymatic sources such as NADPH oxidase, xanthine-oxidase, and by uncoupled nitric oxide (NO) synthase.[2] Although there are differences in the individual sources of superoxide between human arteries and veins,[3,4] there is a strong correlation in superoxide generation between these two vessel types in the human body.[3,4] Therefore, understanding the mechanisms regulating vascular superoxide generation is important for the development of therapeutic strategies targeting intracellular redox signalling.

Endothelial-NO synthase (eNOS) generates NO in the human vascular endothelium by the oxidation of L-arginine.[5] An endogenous L-arginine analogue, asymmetrical dimethylarginine (ADMA), has been associated with impaired endothelial function in humans,[6] suggesting that ADMA may have an effect on eNOS function, whereas clinical evidence suggests that serum ADMA may be a novel cardiovascular risk factor.[7,8]

Despite numerous clinical reports correlating serum ADMA with non-invasive measures of endothelial function,[6] the exact mechanisms by which ADMA modifies NO bioavailability in human vessels are uncertain. Asymmetrical dimethylarginine may act as an eNOS inhibitor, by competitive inhibition of L-arginine oxidation, in a manner analogous to mono-methyl arginine. Alternatively, ADMA may uncouple eNOS, leading not only to the loss of NO but also an increase in superoxide production in the vascular endothelium. In a cell-culture model, ADMA induced the production of superoxide radicals by endothelial cells, suggesting that ADMA has the potential to affect redox-sensitive transcriptional pathways via modulation of superoxide production.[9] However, it is still unclear whether ADMA affects superoxide production by modulating eNOS coupling or by affecting the activity of other enzymatic sources of superoxide.

In the present study, we sought to examine the associations between serum ADMA levels, NO-mediated endothelial function, eNOS coupling, and vascular superoxide generation in human arteries and veins obtained from patients with coronary atherosclerosis.

Methods

Study subjects

We initially screened 303 patients with coronary artery disease (CAD) undergoing elective coronary artery bypass surgery (CABG) at the John Radcliffe Hospital, Oxford, UK, during the years 2004–06 (and belonged to a cohort recruited for a previous study),[10] of which 219 met the inclusion criteria and agreed to participate. Of these patients, 18 subjects were deemed ineligible for inclusion due to inadequate tissue sample size, and the final study population was 201 subjects. Exclusion criteria were any inflammatory, infective, liver or renal failure, malignancy, acute coronary event during the last 2 months, or clinically overt heart failure. Patients receiving non-steroidal anti-inflammatory drugs, dietary supplements of folic acid, or antioxidant vitamins were also excluded. All patients discontinued nitrate treatment at least 24 h before samples intake. Demographic characteristics of the patients are presented in *Table 1*. Importantly, none of the participants was receiving fibrates, fish oils, niacin, or other vasoactive agents. The study was approved by the local Research Ethics Committee, and each patient gave written informed consent.

Plasma and tissue samples

Blood samples were obtained immediately before surgery, after overnight fasting. Samples were centrifuged at 2500 rpm for 10 min, and serum or plasma was stored at −80°C until assayed. Samples of saphenous vein (SV) and internal mammary artery (IMA) were obtained at the time of CABG surgery, as described previously.[11,12] Paired vessel segments were snap-frozen and stored at −80°C, for later analysis, or were transferred to the laboratory for functional studies within 30 min, in ice cold Krebs–Henseleit buffer.[11,12]

Cell culture experiments

Human umbilical vein endothelial cells (HUVECs) were cultured at 37°C in a humidified 5% CO atmosphere in EGM-2 bullet kit medium (Cambrex, cat no. CC-3162). Human umbilical vein endothelial cells between passages 3 and 6 were used for experiments. Prior to experiments, the cells were incubated for 48 h in medium containing either ADMA 1 mM or no additional ADMA (control). The cells were grown in glass cover slips, which were transferred to the luminometry vial for superoxide measurements at the end of the incubation period.

Determination of superoxide production

Vascular superoxide production was measured in paired segments of SVs and IMAs using lucigenin-enhanced chemiluminescence, as described previously.[12,13] Vessels were opened longitudinally to expose the endothelial surface and equilibrated for 20 min in oxygenated (95% O_2/5% CO_2) Krebs-HEPES buffer (pH = 7.4) at 37°C. For the cell culture experiments, the HUVEC-confluent cover slips were transferred into the luminometry vial, and the experiments were carried out as for the vascular segments. Lucigenin-enhanced chemiluminescence was measured using low-concentration lucigenin (5 μM).[2,12] As a measure of NADPH-oxidase activity, we determined NADPH-stimulated superoxide production in the presence of NADPH 100 μM, as we have described previously.[3] As a measure of eNOS coupling in SV and IMA segments, we determined NOS-derived superoxide production, which was estimated as the difference in superoxide production following 20 min incubation with the NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME; 100 μM). As lucigenin-enhanced chemiluminescence favours redox cycling of lucigenin at high concentrations (250 μM),[13] we used low lucigenin concentration (5 μM) as suggested in the literature.[13] In addition, lucigenin redox recycling is detectable even at lucigenin concentrations as low as 5 μM when using NADH as a substrate, a phenomenon that is however, further reduced when NADPH is used as a substrate,[14] as we do in our studies. As we have previously shown,[3,4] NADPH-stimulated superoxide measurement in intact vessels is closely associated with the NADPH-stimulated superoxide measurement in vascular homogenates or by using superoxide dismutase inhibitable ferrocytochrome *c* reduction. Finally, we did not use any NADPH oxidase inhibitor such as apocynin, as that was recently shown not to be a specific NADPH oxidase inhibitor in the vascular wall,[15] and it predominantly acts as non-specific antioxidant.

Table 1 Demographic characteristics of patients

	ADMA (tertiles)			
	Low	**Medium**	**High**	***P*-value**
Serum ADMA (μmol/L)[a]	0.21 [0.20–0.35]	0.59 [0.49–0.70]	1.1 [0.9–1.5]	0.0001
Males/females *n* (%)	58 (87)/9 (123)	57 (86)/10 (14)	57 (86)/10 (14)	0.722
Age (years)	67.2 ± 7.8	66.0 ± 8.9	66.3 ± 8.4	0.728
Risk factors				
Hypertension (%)	48 (71)	50 (75)	45 (69)	0.787
Hypercholesterolaemia (%)	54 (81)	49 (73)	50 (74)	0.563
Smokers (ex/current) (%)	38 (57)/7 (10)	35 (51)/9 (13)	40 (60)/8 (12)	0.917
Diabetes mellitus (%)	16 (24)	17 (25)	18 (27)	0.644
Ejection fraction of the left ventricle (%)	51.9 ± 11.0	52.8 ± 10.0	49.9 ± 12.1	0.341
Extend of CAD (%)				
One-vessel disease	2 (3)	2 (3)	2 (3)	0.384
Two-vessel disease	20 (30)	18 (27)	15 (22)	
Three-vessel disease	45 (67)	47 (71)	52 (77)	
Body mass index (kg/m^2)	27.9 ± 3.7	27.7 ± 4.4	27.1 ± 5.5	0.708
Triglycerides (mmol/L)[a]	1.6 [1.1–2.0]	1.40 [1.0–2.0]	1.3 [0.9–1.9]	0.322
Cholesterol (mmol/L)	4.2 ± 0.8	4.1 ± 0.7	3.8 ± 1.1	0.154
High-density lipoprotein (mmol/L)	1.2 ± 0.1	1.1 ± 0.2	1.1 ± 0.3	0.862
Serum creatinine (μmol/L)	108.1 ± 11.5	102.6 ± 26.3	101.8 ± 4.7	0.715
Plasma ox-LDL (IU/L)[a]	40.1 [30.1–49.8]	44.8 [34.6–53.6]	49.3 [32.2–68.3]	0.014
Medication (%)				
Statins	64 (95)	62 (92)	62 (93.1)	0.792
ACEI/ARB	51 (75)	44 (66)	49 (73)	0.790
Calcium channel blockers	26 (38)	19 (29)	25 (37)	0.335
β-blocker	55 (81)	61 (91)	55 (82)	0.153
Aspirin	60 (90)	57 (86)	61 (91)	0.631
Clopidogrel	18 (26)	16 (24)	19 (28)	0.906

CAD, coronary artery disease; ACEI, angiotensin-converting enzyme-inhibitors; ARB, angiotensin II receptor blockers; ADMA, asymmetrical dimethylarginine; ox-LDL, oxidized low-density lipoprotein. Values expressed as mean ± SD.

[a]Values expressed as median [25–75th percentile]. There was no significant difference in any of the baseline clinical characteristics, medications, or serum lipids between the three groups. In addition, there was no association between serum ADMA (as a continuous variable) and any of the variables listed above (except for ox-LDL).

Vasomotor studies

Endothelium-dependent and endothelium-independent dilatations were assessed in SV rings obtained at the time of CABG, using isometric tension studies.[2,12] Briefly, vascular segments were transferred to a laboratory in ice-chilled Krebs–Hensleit buffer within 30 min from harvesting and were used immediately for the organ bath experiments. These vascular segments were carefully harvested by using a no-touch technique, and they were obtained before distension (to minimize the possibility of any damage to the endothelium or to the vascular wall). Rings with obvious damage of the vascular wall due to surgical manipulations were discarded. At the beginning of each experiment, the vessels were equilibrated in the organ bath for 60 min, during which we aimed to achieve a final resting tension of 3 g (by adding 1 g every 5 min after the 20th minute). The viability of the rings used was tested by exposing them to the Krebs–Hensleit buffer containing potassium chloride (KCl 60 mM) for 5 min. Four rings from each vessel were pre-contracted with phenylephrine (3×10^{-6} M), and then endothelium-dependent relaxations were subsequently quantified using acetylcholine (ACh, 10^{-9}–10^{-5} M) and bradykinin (Bk, 10^{-9}–3×10^{-6} M). Finally, relaxations to the endothelium-independent NO donor sodium nitroprusside (SNP, 10^{-10}–10^{-6} M) were evaluated in the presence of L-NAME (100 μM), as described previously.[2,12]

Determination of asymmetrical dimethylarginine and oxidized-low-density lipoprotein levels

Serum levels of ADMA were determined by enzyme-linked immunosorbent assay (ELISA, DLD Diagnostika, Hamburg, Germany). The sensitivity of this assay is 0.05 μmol/L, and its specificity for ADMA is 100%. The results from this assay are closely correlated with those derived from LC-MS-MS and GC–MS ($r > 0.9$). Similarly, serum levels of ox-LDL were measured by ELISA (Mercodia, Sweden).

Statistical analysis

All variables were tested for normal distribution by using the Kolmogorov–Smirnov test. Normally distributed variables are presented as mean ± SD, whereas non-normally distributed variables were

log-transformed for analysis and are presented as median (25–75th percentiles) and range. Power calculations were based on previous results from our group,[16] which showed that a sample of 15 patients per group was able to detect a 40% difference and 65 patients a 20% difference in maximum relaxations in response to ACh between groups, with a power of 90% and $\alpha = 0.05$. Comparisons of baseline and demographic characteristics between patients in the respective tertiles of ADMA were performed by χ^2 test for categorical variables and by one-way analysis of variance (ANOVA) followed by Bonferroni *post hoc* correction for individuals between group comparisons. To examine the association between ADMA tertiles and vasorelaxation in response to ACh, Bk, and SNP, we used two-way ANOVA for repeated measures, where we examined the interaction between ADMA levels (as a continuous variable) and ACh, Bk, or SNP concentration, respectively, in their effect on vasorelaxation. The concentration of each vasodilator and serum ADMA levels were used as fixed-effects covariates. As each vasorelaxation experiment was performed on the same vascular tissue sample, the analysis had one level of within-patient correlations (for multiple concentrations). Correction for possible intra-group correlations was performed by the Greenhouse–Geisser method.

Bivariate analysis was performed, and Pearson's r or Spearman's ρ coefficient was calculated. Multiple linear regression was used to examine the association of serum ADMA with vascular superoxide, L-NAME-induced change of vascular superoxide, or maximum relaxations of SV to ACh. As independent variables, we used serum ADMA as well as age, gender, diabetes mellitus, smoking, dyslipidaemia, hypertension, and body mass index. The selection of these potential predictors was based on the underlying known biology, since previous reports suggest that they may affect the vascular redox state and endothelial function. The main rationale of each model was to test whether the association observed between serum ADMA (independent variable) and vascular O2- or endothelial function (dependent variable) was independent of other clinical variables, which may also have an effect on the dependent variable. The models were cross-validated by estimating the adjusted R^2 values, derived by Stein's formula,[17] whereas the biological validation was achieved by replicating the results in both SV and IMA separately. All models were tested for basic assumptions as described previously,[18] such as the no perfect multicollinearity, the absence of predictors correlated with known 'external variables' not included into the models, homoscedasticity, the independent errors assumption (tested by Durbin–Watson test), the independence of all the values of the outcome variable, the non-zero variance of all predictors, and the linearity of the modelling associations. All the regression models met these assumptions. *P*-values were two-tailed, and a $P < 0.05$ was considered statistically significant. All statistical analyses were performed using SPSS 12.0.

Results

Patient's baseline and demographic characteristics are presented in *Table 1*. There was no significant association between serum ADMA and any of these baseline/demographic characteristics, either when ADMA was used as a continuous variable or as tertiles of its serum levels (*Table 1*).

Associations between serum asymmetrical dimethylarginine and vascular nitric oxide bioavailability

Serum ADMA levels were inversely associated with the maximum endothelial-dependent relaxations in response to both ACh ($r = -0.32$, $P = 0.005$) and Bk ($r = -0.37$, $P = 0.001$), but not to the endothelium-independent agonist SNP ($r = 0.03$, $P = 0.8$). In multivariable analysis, ADMA was associated with maximum vasorelaxation in response to ACh and Bk, independent of age, gender, and atherosclerosis risk factors (*Table 2*). To further evaluate the relationship between serum ADMA and vasomotor responses, we examined the effect of serum ADMA on the vasorelaxation dose–response curves to ACh and Bk, by using serum ADMA as a continuous variable. The interaction between ADMA levels and vasodilator concentration in their effect on vasorelaxation was significant for ACh and Bk but not for SNP, whereas for presentation purposes, the vasorelaxation curves are presented according to the tertiles of serum ADMA (*Figure 1*).

Asymmetrical dimethylarginine and oxidative stress

Vascular superoxide generation in SVs was significantly correlated with that in IMAs ($r = 0.333$, $P = 0.025$). Similarly, L-NAME-inhibitable superoxide in SVs was strongly correlated with that in IMAs ($r = 0.526$, $P = 0.0001$), and a significant association of vascular NADPH-stimulated superoxide was also observed between SV and IMA ($r = 0.395$, $P = 0.0001$). These findings suggest that vascular superoxide generation follows a similar pattern across the entire human vasculature.

Serum ADMA levels correlated significantly with plasma ox-LDL ($r = 0.353$, $P = 0.0001$), whereas there was a significant difference in ox-LDL levels between the three groups defined by serum ADMA tertiles (*Table 1*), suggesting an association between ADMA and systemic oxidative stress. To further investigate the

Table 2 Results of linear regression for maximum vasorelaxation to acetylcholine and bradykinin

	Model R^2	Stand. *B*	*P*-value
Predictors of max. relaxations to ACh	0.255		
Log [ADMA]		0.266	0.025
Smoking		0.084	0.484
Diabetes mellitus		0.164	0.155
Hypertension		−0.138	0.255
Dyslipidaemia		0.242	0.047
Body mass index		0.006	0.957
Female gender		0.024	0.838
Age		0.352	0.007
Predictors of max. relaxations to Bk	0.170		
Log [ADMA]		0.216	0.028
Smoking		0.133	0.169
Diabetes mellitus		0.074	0.439
Hypertension		0.194	0.055
Dyslipidaemia		0.207	0.038
Body mass index		0.068	0.484
Female gender		0.183	0.063
Age		0.130	0.203

ADMA, asymmetrical dimethylarginine; ACh, acetylcholine; Bk, bradykinin.

effect of ADMA on the vascular redox state, we determined vascular superoxide generation in paired samples of SVs and IMAs obtained from these patients. We found that ADMA was positively correlated with vascular superoxide generation in both SV ($r = 0.231$, $P = 0.013$) and IMA ($r = 0.339$, $P = 0.0001$) (*Figure 2*).

In multivariable analysis, serum ADMA was associated with vascular superoxide generation in SV and IMA, independent of age, gender, and atherosclerosis risk factors (*Table 3*).

To further investigate whether serum ADMA might be associated with specific sources of vascular superoxide, we first examined its effect on eNOS-derived vascular superoxide, as a measure of eNOS coupling. Serum ADMA was significantly correlated with the L-NAME-induced change in vascular superoxide in both SV ($r = -0.317$, $P = 0.001$) and IMA ($r = -0.372$, $P = 0.0001$) (*Figure 3*), suggesting that serum ADMA is closely associated with eNOS coupling.

In multivariable analysis, serum ADMA was associated with vascular L-NAME-inhibitable superoxide generation in SV and IMA, independent of age, gender, and atherosclerosis risk factors (*Table 3*).

Since NADPH oxidase is a major source of vascular superoxide in human vessels, we also examined whether the NADPH-

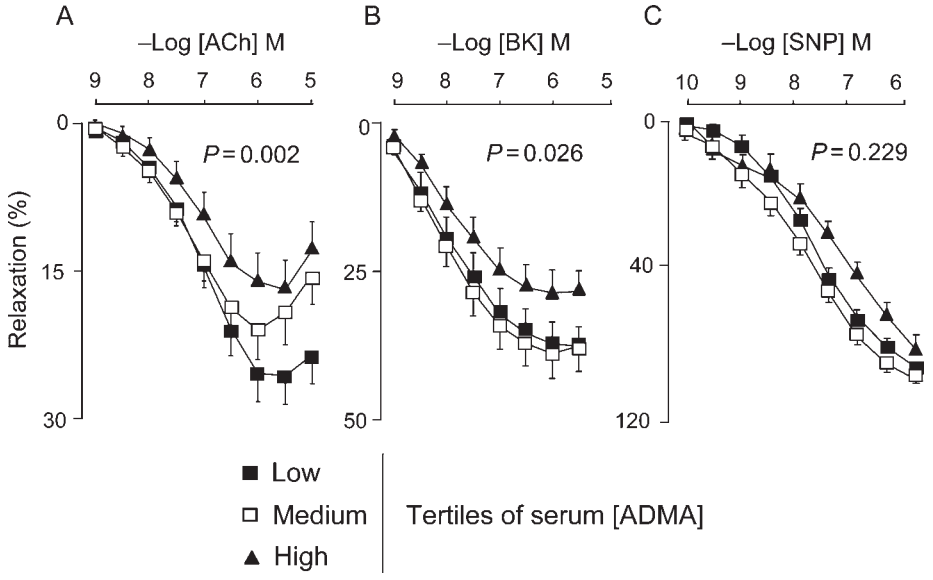

Figure 1 Serum asymmetrical dimethylarginine [(ADMA), by tertiles] and vasorelaxation of saphenous vein segments in response to acetylcholine (*A*), bradykinin (*B*), and sodium nitroprusside (*C*). *P*-values derived by two-way analysis of variance for repeated measurements, by using serum ADMA as a continuous variable.

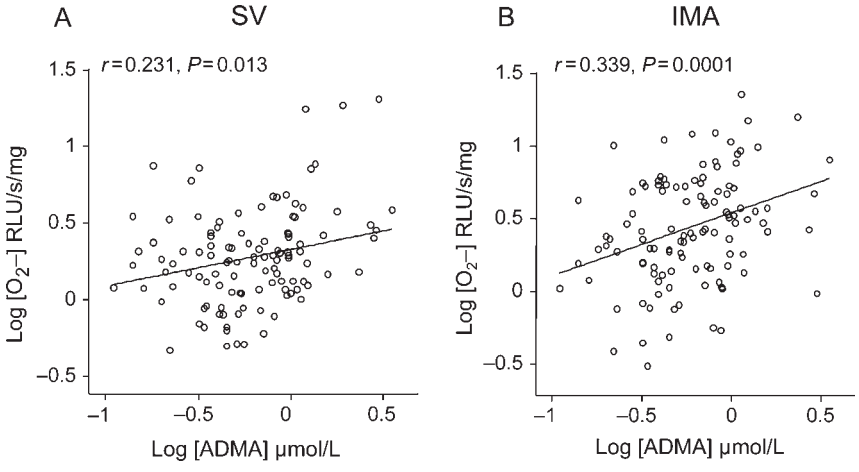

Figure 2 Circulating asymmetrical dimethylarginine (ADMA) as a determinant of vascular superoxide in saphenous veins (*A*) and internal mammary arteries (*B*) from patients with coronary artery disease.

Table 3 Results of linear regression for vascular superoxide (O_{2}^{-}) generation

	SV			IMA		
	Model R^2	Stand. *B*	*P*-value	Model R^2	Stand. *B*	*P*-value
Predictors of basal O2-	0.170			0.224		
Log [ADMA]		0.216	0.028		0.395	0.000
Smoking		0.133	0.169		0.008	0.938
Diabetes mellitus		0.074	0.439		−0.105	0.268
Hypertension		0.194	0.055		0.141	0.166
Dyslipidaemia		0.207	0.038		0.055	0.562
Body mass index		0.068	0.484		0.024	0.796
Female gender		0.183	0.063		−0.032	0.744
Age		0.130	0.203		−0.208	0.047
Predictors of L-NAME-inhibitable O2-	0.246			0.197		
Log [ADMA]		−0.214	0.039		−0.368	0.001
Smoking		−0.144	0.164		0.029	0.794
Diabetes mellitus		0.060	0.547		0.038	0.716
Hypertension		−0.219	0.038		−0.158	0.188
Dyslipidaemia		−0.027	0.789		0.028	0.799
Body mass index		−0.138	0.169		−0.018	0.867
Female gender		−0.392	0.000		−0.007	0.948
Age		−0.109	0.308		0.235	0.057

ADMA, asymmetrical dimethylarginine; SV, saphenous vein; IMA, internal mammary artery.

stimulated vascular superoxide was associated with serum ADMA. However, we observed no significant association between serum ADMA and NADPH-stimulated vascular superoxide generation, in either SV ($r = -0.01$, $P = 0.919$) or IMA ($r = 0.180$, $P = 0.620$), as demonstrated in *Figure 3*.

To examine whether the association between ADMA and vascular superoxide generation is causal, we incubated HUVECs with ADMA 1 mM for 48 h and examined the effect on the overall superoxide generation. We observed that HUVECs incubated with ADMA had significantly higher total superoxide generation (10.32 ± 1.42 RLU/s/cell count) compared to control cells (4.49 ± 0.95 RLU/s/cell count, $P = 0.004$), suggesting a causal effect of ADMA on endothelium-derived superoxide (*Figure 4*).

Discussion

In this study, we define new associations between serum ADMA levels, endothelial function, and vascular oxidative stress in patients with CAD. We report several important observations. First, serum ADMA is inversely related to vascular NO bioavailability in human vessels. Secondly, serum ADMA is correlated with, and is an independent predictor of, vascular superoxide generation. Finally, serum ADMA is a determinant of eNOS coupling, but is not associated with vascular NADPH-stimulated superoxide in intact human vessels. Taken together, the results of this study demonstrate that ADMA is an independent determinant of the vascular redox state and endothelial function in patients with CAD, with effects on eNOS coupling and NO bioavailability.

Clinical evidence suggest that serum ADMA levels are inversely associated with endothelial function, evaluated by non-invasive means, in healthy individuals,[6] in patients with coronary atherosclerosis,[19,20] and in subjects with multiple risk factors.[19,21] Indeed, in a recent large-scale study by Juonala *et al.*,[6] it was clearly documented that serum ADMA is inversely correlated with flow-mediated dilation in the brachial artery (a measure of endothelial function), independent of classic risk factors for atheroscleoris. Furthermore, serum ADMA levels appear to be associated with increased cardiovascular risk in the general population[8,22] and in high-risk individuals,[23] whereas it has a predictive value in patients with coronary atherosclerosis,[7] especially after angioplasty.[24] However, these clinical observations have not been supported by strong mechanistic data relating serum ADMA with endothelial function or vascular disease risk in humans. We now show for the first time that the serum ADMA is not only associated with decreased NO bioavailability (as demonstrated by the *ex vivo* vasorelaxation of vascular segments in response to ACh or Bk), but also with increased vascular oxidative stress, demonstrated by direct measures or vascular superoxide production.

The association of ADMA with oxidative stress status is now well documented. Evidence suggests that ADMA is synthesized by redox-sensitive methylating enzymes such as S-adenosylmethionine-dependent protein arginine N-methyltransferases[25] that methylate arginine residues in proteins to form ADMA. Therefore, ADMA synthesis is enhanced under conditions of increased oxidative stress. In addition, the enzyme responsible for ADMA degradation, dimethylarginine dimethylamino-hydrolase, is also redox-sensitive,[26] as its activity is decreased in the presence of increased oxidative stress status. Therefore, oxidative stress increases ADMA synthesis and decreases its degradation, partly

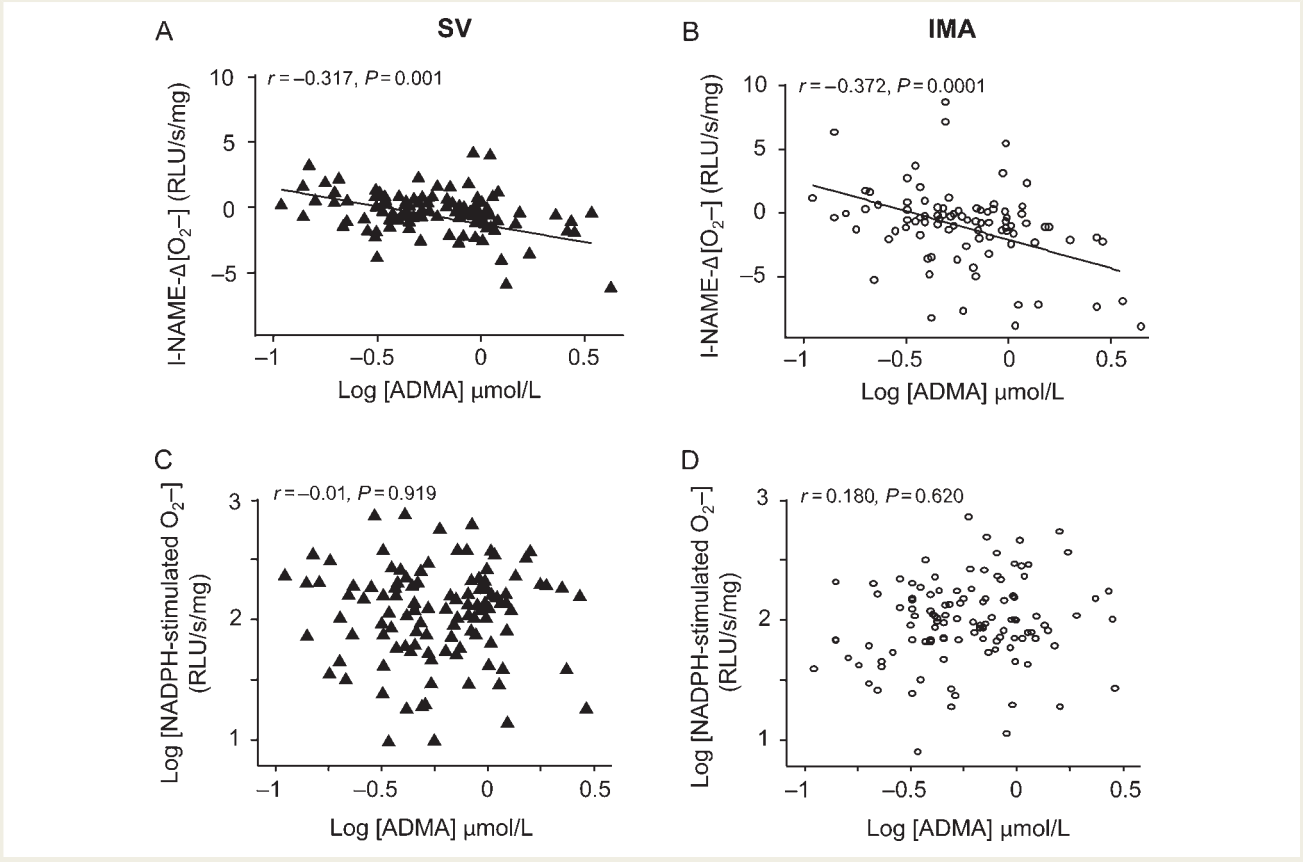

Figure 3 Serum asymmetrical dimethylarginine (ADMA) was associated with the L-NAME-inhibitable fraction of vascular superoxide (O2-) in both saphenous veins (SV, *A*) and internal mammary arteries (IMA, *B*) from patients with coronary artery disease. However, there was no association between serum ADMA and the NADPH-stimulated vascular O2- in vessels from the same patients (*C* and *D* for SV and IMA, respectively).

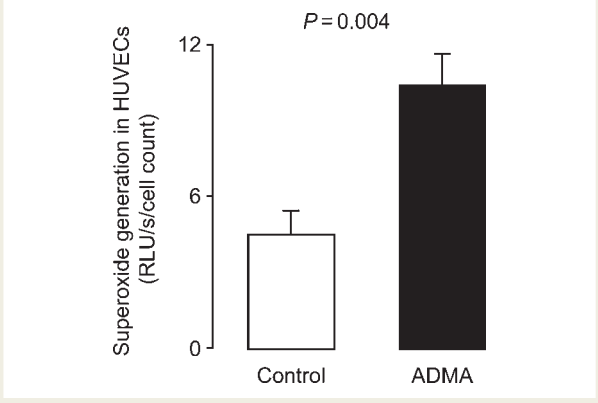

Figure 4 Incubation of human umbilical vein endothelial cells (HUVECs) with asymmetrical dimethylarginine 1 mM for 48 h induced a significant elevation of superoxide production by these cells; $n = 3$ experiments.

explaining the clinical observations that serum ADMA is associated with increased systemic markers of oxidative stress in patients with multiple risk factors for atherosclerosis or CAD.[27] However, the mechanistic relationship between serum ADMA and oxidative stress in human vascular disease has remained unclear, as there is evidence that ADMA itself may affect oxidative stress status. A causal relationship between ADMA and endothelium-derived superoxide radical generation has been suggested by *in vitro* models.[9] Incubation of endothelial cells with ADMA significantly elevated superoxide radical generation, a finding confirmed in isolated arterioles from rat gracilis muscle, where ADMA was found to increase superoxide radical generation *ex vivo*.[28] In line to these observations, it was also demonstrated that ADMA accelerates cell senescence in endothelial cell culture, probably by increasing oxygen radical formation and by inhibiting NO elaboration.[29] The known action of ADMA as an eNOS inhibitor could be due to the inhibition of cellular L-arginine uptake by endothelial cells, leading to intracellular L-arginine deficiency or to direct inhibition of L-arginine oxidation by the eNOS enzyme. Equally, ADMA could induce eNOS uncoupling, either through substrate reduction or through a direct effect on eNOS catalysis. The uncoupled form of eNOS then becomes a source of superoxide radicals instead of NO. Alternatively, the effect of ADMA on endothelial superoxide generation in animal and cell culture models could be due to the activation of other enzymatic sources of superoxide radicals in endothelial cells such as NADPH oxidases. Previous *in vitro*

experiments with purified neuronal NOS protein suggested that ADMA may act as a direct NOS inhibitor rather than as an inducer of NOS uncoupling,[30] although there have been no studies examining its effect on eNOS. Furthermore, there have been no studies examining the association between ADMA and eNOS coupling or the activity of other enzymatic sources of vascular superoxide, such as the NADPH oxidases in human vasculature.

In the present study, we found no association between serum ADMA and NADPH-stimulated superoxide in intact human arteries and veins. However, we did find a strong association between serum ADMA and eNOS coupling in both human arteries and veins, measured by the LNAME-inhibitable fraction of vascular superoxide production. Despite these significant associations, the clinical part of this study does not document any causal association between ADMA and vascular redox in humans. In line to these observations, we performed *in vitro* experiments, and we demonstrated that incubation of HUVEC with ADMA induces a significant elevation of total superoxide generation by these cells. This could be due to a direct effect of ADMA on enzymatic coupling of eNOS, or it could be due to the antagonism between ADMA and L-arginine, for cellular uptake by endothelial cells. Further to the causal effect of ADMA on superoxide generation by endothelial cells, at a clinical level, systemic oxidative stress could lead to increased systemic ADMA levels (since serum ADMA is positively correlated with plasma ox-LDL, a marker of systemic oxidative stress). This systemic oxidative stress could also induce the oxidation of the eNOS co-factor tetrahydrobiopterin in vascular endothelium, and this could then lead to eNOS uncoupling, as we have demonstrated in the same cohort in the past.[10]

In the present study, measurements of redox state and quantification of the various enzymatic sources of superoxide radicals were performed in paired segments of SVs and IMAs. Previous observations from our group have shown that these measures are closely related between these two types of human vessels,[3,11,31] a finding also confirmed in the present study. However, the replication of our findings regarding the associations between serum ADMA and superoxide generation in both vessel types adds more value to the results, making it clear that the links between ADMA and vascular redox are uniform across the entire vascular tree in humans.

The implication of ADMA in the regulation of vascular redox and NO bioavailability in human vessels provides mechanistic explanations of the association of this circulating marker with clinical outcomes in both primary[22] and secondary[24,32] prevention. Indeed, ADMA is an independent predictor of CAD,[22] and it is considered to be a new risk factor for atherosclerosis; therefore, the better understanding of the mechanisms linking this molecule with vascular homeostasis is crucial. Further to the notion that ADMA biosynthetic pathway may be a therapeutic target in atherosclerosis, our findings suggest that targeting vascular redox, eNOS coupling, and endothelial NO bioavailability in human vessels would probably depress the proatherogenic effects ADMA in humans.

In the present study, NO bioavailability was assessed in segments of SV *ex vivo*, and this is a limitation of the study. However, we have demonstrated in the past that SVs[10,12,16] can be used as a reliable model system to evaluate the endothelial function in humans, and the results reflect endothelial function in the entire vasculature. Another limitation of the study comes from the use of lucigenin-enhanced chemiluminescence to evaluate superoxide generation. Lucigenin-enhanced chemiluminescence favours redox cycling of lucigenin at high concentrations (250 μM),[13] a phenomenon that is, however, minimally observed when low lucigenin concentration (5 μM) is used.[13] In addition, lucigenin redox recycling is detectable even at lucigenin concentrations as low as 5 μM when using NADH as a substrate, a phenomenon that is, however, further reduced when NADPH is used as a substrate.[14] Therefore, by using low lucigenin concentrations and NADPH as a substrate in our studies, we reduce these artefacts to the minimum. Although the ability of NADPH to enter intact endothelial cells has been questioned, the NADPH-stimulated superoxide measurements from intact vessels are closely correlated with those derived from homogenates or by using the superoxide dismutase inhibitable ferrocytochrome *c* reduction method.[3] This could be due to the transport of reducing equivalents into cells, thus indirectly increasing intracellular NAD(P)H levels. Alternatively, the membrane-associated NAD(P)H oxidase subunits in vascular cells may have different transmembrane orientations from neutrophil NADPH oxidase, as it has been observed with fibroblasts.[33] Another limitation of the study is that the majority of the participants were receiving angiotensin-converting enzyme-inhibitors, which are known to potentiate the relaxations to Bk and also affect ADMA plasma levels.[34] Finally, most of the participants (71.5%) had multi-vessel disease; therefore, extrapolations of our findings to the general population should be made with caution.

In conclusion, we have demonstrated for the first time that serum ADMA is associated with decreased vascular NO bioavailability in vessels from patients with CAD, with higher systemic oxidative stress, and specifically with increased vascular superoxide generation and eNOS uncoupling. These findings suggest that ADMA may be directly implicated in the regulation of the vascular redox state in human atherosclerosis by affecting superoxide generation and NO bioavailability. Therefore, ADMA may be considered as a possible therapeutic target in human atherosclerosis.

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