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Symmetric dimethylarginine, high-density lipoproteins and cardiovascular disease

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Aims The vascular effects of high-density lipoproteins (HDL) differ under certain clinical conditions. The composition of HDL is modified in patients with chronic kidney disease (CKD). As a consequence, uremic HDL induces endothelial dysfunction. We have previously shown that accumulation of symmetric dimethylarginine (SDMA) in HDL causes these adverse effects of HDL in CKD. The aim of the study is to determine the impact of the accumulation of SDMA on the association between HDL and mortality.

Methods and results Mortality, renal function, serum SDMA and HDL-cholesterol (HDL-C) were assessed in the LURIC study including 3310 subjects undergoing coronary angiography. All-cause mortality was 30.0% during median follow-up of 9.9 years. Serum SDMA levels significantly predicted all-cause and cardiovascular mortality, and were significantly correlated with SDMA accumulation in HDL. Notably, higher serum SDMA was independently associated with lower cholesterol efflux ($P = 0.004$) as a measure of HDL functionality. In subjects with low SDMA levels, higher HDL-C was associated with significantly lower mortality. In contrast, in subjects with high SDMA, HDL-C was associated with higher mortality. These findings were confirmed in 1424 participants of the MONICA/KORA S3 cohort. Of note, we derived an algorithm allowing for calculation of biologically effective HDL-C' based on measured HDL-C and SDMA. We corroborated these clinical findings with *in vitro* evidence showing that SDMA accumulation abolishes the anti-inflammatory and regenerative properties of HDL.

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Conclusion The data identify SDMA as a marker of HDL dysfunction. These findings highlight on the pivotal role of SDMA accumulation in HDL as a mediator of pre-mature cardiovascular disease in patients with CKD.

Keywords High-density lipoproteins • Kidney disease • Cardiovascular risk • Symmetric dimethylarginine

Introduction

Cardiovascular disease (CVD) represents the major cause of death in Europe.[1] Amongst other cardiovascular (CV) risk factors, chronic kidney disease (CKD) represents one of the strongest and independent risk factors for CVD.[2–5] Interestingly, even mild kidney dysfunction substantially increases the cardiovascular burden.[6–9] Notably, the demographic change in Europe with a growing proportion of elderly with high prevalence of diabetes mellitus and arterial hypertension leads to a steadily increasing incidence and prevalence rate of CKD. The prevalence of CKD in the 6th decade of life is higher than 30%.[10] This inevitably moves CKD in the focus of cardiovascular medicine.

In healthy subjects, high-density lipoproteins (HDL) exert vasoprotective effects by stimulating endothelial nitric oxide production, reducing endothelial production of reactive oxygen species (ROS) and preventing pro-inflammatory responses of the endothelium.[11–13] Observational studies confirm that high concentrations of HDL-cholesterol (HDL-C) are associated with improved cardiovascular outcome.[14,15] Interestingly, under several disease conditions such as CKD, diabetes mellitus, and coronary artery disease, HDL may lose its vasoprotective properties.[16–18] Of note, a recent register study of 1 764 986 US-Veterans found a U-shaped relationship between HDL-C and mortality. The association of HDL-C ≥50 mg/dL (>1.3 mmol/L) with mortality became more prominent the more eGFR decreased.[19]

In CKD patients, several factors can modify the composition of the HDL particle in different ways. Uremic toxins, increased oxidative stress as well as a pro-inflammatory micro-environment contribute to a remodelling of the HDL particle, thereby altering the composition of the proteome and lipidome of HDL and inducing post-translational modifications of HDL's protein cargo.[20] The accumulation of uremic toxins such as dimethylarginines with kidney impairment might therefore play a key role in the HDL dysfunctionality. The dimethylarginines, asymmetric dimethylarginine (ADMA) and symmetric dimethylarginine (SDMA) are predictors of atherosclerosis and cardiovascular events in CKD patients as well as in the general population.[21–23] Whereas ADMA exerts its negative effects on the vascular wall as an endogenous inhibitor of nitric oxide synthesis, the vascular effects of SDMA are still not completely understood.[24] Recently, our group identified the accumulation of SDMA in the HDL particle of patients with impaired kidney function being responsible for the adverse effects of HDL in CKD.[25] Notably, we could not detect any ADMA in the HDL fractions. This accumulation of SDMA in the HDL particle transforms HDL into a noxious molecule inducing endothelial dysfunction, pro-inflammatory activation and hypertension. These deleterious effects of HDL on endothelial cells are mediated via Toll-like receptor-2.[25] We could document the adverse endothelial effects of HDL also in children with CKD, in whom cardiovascular risk factors such as smoking, hypertension, diabetes mellitus, and dyslipidaemia were not yet present,[26] highlighting on impaired renal function itself as the culprit, which modifies the vascular properties of HDL.

However, the clinical relevance of these experimental findings remains unknown. Therefore, in the present study, we examined the effect of SDMA on the long-term prognostic value of HDL-C.

Methods

Study populations

Between 1997 and 2000, the Ludwigshafen Risk and Cardiovascular Health (LURIC) study enrolled 3316 patients undergoing coronary angiography.[27] The study design and the examinations of the whole cohort at baseline have been previously described.[27] The median follow-up time was 9.9 years. Taken together 30.0% of the participants of the LURIC study died ($n = 995$) during follow-up time. Cardiovascular mortality was defined as sudden cardiac death, fatal myocardial infarction, death due to congestive heart failure, or death immediately after intervention to treat coronary artery disease and fatal stroke.

The population-based MONICA/KORA S3 study comprises 4856 participants being recruited in 1994/95 in the German city Augsburg region including the city of Augsburg and the two adjacent counties Augsburg and Aichach-Friedberg as part of the international World Health Organization (WHO) MONItoring of trends and determinants in CArdiovascular Diseases (MONICA) project, which is now continued in the framework of KORA (Co-poperative health research in the region of Augsburg). Details of the MONICA/KORA S3 study have been previously described in detail.[28,29] A total of $n = 1678$ men and women participated in an echocardiographic substudy.[30,31] Of these, 1424 participants, in whom SDMA and ADMA were available at baseline, were included in the present analysis. The median follow-up time was 16.8 years. In summary 18.0% of the participants ($n = 256$) included in the present analysis died during follow-up period. Studies were performed in accordance with the Declaration of Helsinki and have been approved by the local authorities (Rhineland-Palatinate Chamber of Physicians, Bavarian Chamber of Physicians, Germany). Written informed consent of the participants was obtained.

Laboratory measurements in Ludwigshafen risk and cardiovascular health and MONICA/KORA S3

Detailed procedures of laboratory measurements in the LURIC and MONICA/KORA S3 study have been described.[18,28,29,32] SDMA and ADMA measurements in the LURIC study have been performed by reversed-phase HPLC.[33] SDMA and ADMA measurements in the MONICA/KORA S3 study have been performed by using a fully validated high-throughput LC-MS/MS assay.[34]

Table 1 Association of cardiovascular risk factors and markers of disease severity with SDMA and ADMA concentrations in the LURIC study

	SDMA[a] (μmol/L)	Mean difference[b] (%)	*P*	ADMA[a] (μmol/L)	Mean difference[b] (%)	*P*
Acute coronary syndrome						
Yes	0.65	+2.5	0.090	0.85	+1.1	0.190
No	0.63			0.84		
Diabetes						
Yes	0.64	-0.2	0.855	0.85	+1.2	0.059
No	0.64			0.84		
Smoking						
Yes	0.64	-1.1	0.366	0.86	+2.2	0.001
No	0.64			0.84		
Gender						
Female	0.64	-1.4	0.267	0.85	-0.1	0.828
Male	0.65			0.85		
eGFR						
≥ 90 mL/min	0.46			0.77		
60-89 mL/min	0.56	+22.2	<0.001	0.83	+7.5	<0.001
< 60 mL/min	0.90	+95.5	<0.001	0.94	+21.2	<0.001

ADMA, asymmetric dimethylarginine; SDMA, symmetric dimethylarginine.
[a]Estimated marginal means as calculated in a generalized linear model, adjusted for age, sex, high-sensitive C-reactive protein (CRP), eGFR, body mass index, diabetes, smoking status, acute coronary syndrome, lipid-lowering therapy, Friesinger score, haemoglobin, albumin.
[b]For the comparison with the first category of each variable.

Measurement of the cholesterol efflux capacity in Ludwigshafen risk and cardiovascular health

Cholesterol efflux capacity was measured in 2472 participants of the LURIC study as described.[35] Briefly, J774 cells, derived from a murine macrophage cell line, were plated and radiolabelled with 2 μCi of 3H-cholesterol per millilitre. Cells were incubated with 0.3 mM cAMP (C3912, Sigma-Aldrich) to upregulate ABCA1. Subsequently, efflux medium containing 2.8% apolipoprotein B–depleted serum was added for 4 h. All steps were performed in the presence of 2 μg/mL acyl–coenzyme A: cholesterol acyltransferase inhibitor (Sc-215839A, Santa-Cruz Biotechnology). Liquid scintillation counting was used to quantify the efflux of radioactive cholesterol from the cells. Percent efflux was calculated by the following formula: (microcuries of 3H-cholesterol in medium containing 2.8% apolipoprotein B-depleted serum—microcuries of 3H-cholesterol in serum-free medium)/microcuries of 3H-cholesterol in cells extracted before the efflux step × 100. To correct for interassay variation across plates, a pooled serum control was included on each plate. Values for samples from patients are given in percentage of this control (% C). All assays were performed in triplicate.

Endothelial assays

Detailed description is presented in Supplementary material online.

Statistical analysis

Detailed description is presented in Supplementary material online. In brief, in LURIC the association between HDL-C and SDMA with all-cause and cardiovascular mortality as end-points has been examined by using Cox regression analyses including 1st-order interaction terms between HDL-C × SDMA. In endothelial assays, to determine the functionality of HDL, statistical differences were examined by one-way analysis of variance followed by Dunnett's multiple comparison *post hoc* test. Statistical analyses were carried out with SPSS 21.0 and R project.

Results

The baseline characteristics of the participants of the Ludwigshafen risk and cardiovascular health study (LURIC study) with 3316 participants are presented in Supplementary material online, *Table S1* according to quartiles of SDMA and ADMA serum levels. As shown in see Supplementary material online, *Figure S1*, both SDMA and ADMA significantly and inversely correlate with kidney function (i.e. eGFR creatinine-cystatin C by using CKD-EPI equation). However, the correlation between eGFR and SDMA was much stronger as compared with eGFR and ADMA (R^2 0.542 and R^2 0.175, respectively). In contrast to ADMA, SDMA serum levels were only associated with kidney function and not with other confounding factors such as diabetes mellitus or smoking (*Table 1*). In subjects with an eGFR below 60 mL/min, SDMA concentrations were almost twice compared with those with an eGFR within the normal range.

After a median follow-up time of 9.9 years in the LURIC study, 30.0% ($n = 995$) of the enrolled participants died. More than half of deaths were caused by cardiovascular or cerebrovascular events (66.5%, $n = 622$). Survival analyses revealed both SDMA and ADMA as significant predictors for all-cause as well as cardiovascular mortality (see Supplementary material online, *Figure S2*).

Next, we determined the relationship between total SDMA serum levels and SDMA concentration in HDL from 35 patients with different degrees of kidney impairment (*Figure 1A*). We found a significant association between SDMA serum levels und SDMA in the HDL fractions ($R^2 = 0.66$, $P < 0.0001$). We therefore tested the hypothesis

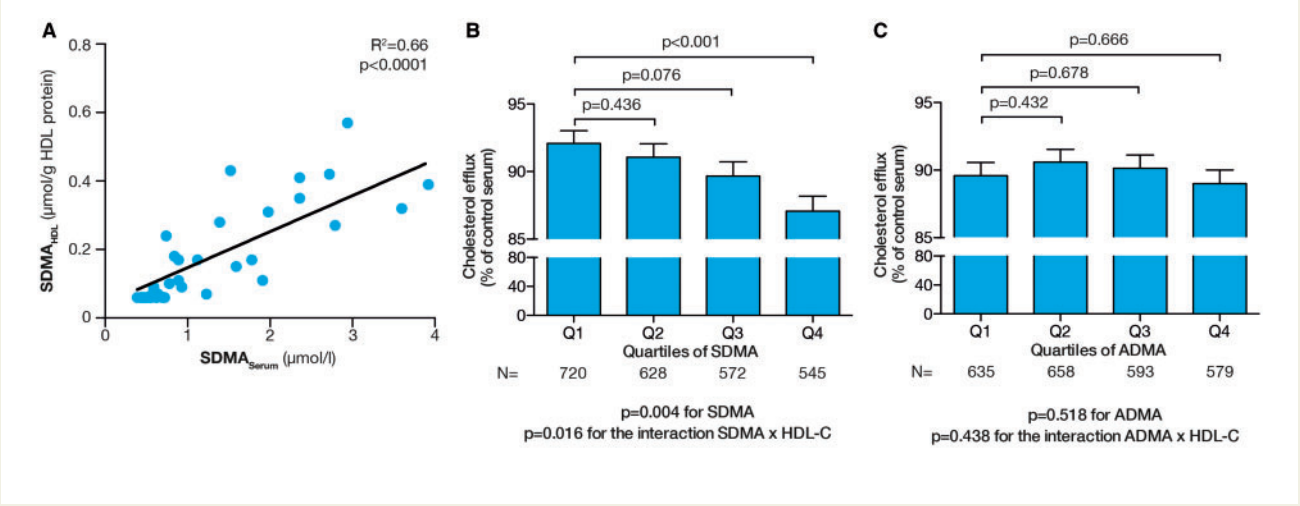

Figure 1 (*A*) Bivariate correlation between total serum SDMA ($SDMA_{Serum}$) and SDMA in HDL ($SDMA_{HDL}$), both determined with HPLC/MS-MS, in 35 patients with varying eGFR. (*B and C*) Multivariable adjusted estimated marginal means of cholesterol efflux in sub-groups of participants of the LURIC study ($n = 2472$) according to quartiles of (*B*) SDMA or (*C*) ADMA, respectively. Results are adjusted for age, gender, acute coronary syndrome, Friesinger score, body-mass index, glycated haemoglobin, smoking, lipid-lowering therapy, cystatin C, high sensitivity CRP, and mean systolic blood pressure. SDMA, symmetric dimethylarginine; HDL, high-density lipoproteins; ADMA, asymmetric dimethylarginine.

that SDMA serum levels might also be a surrogate for the functional properties of HDL, and examined the association between SDMA and ADMA with cholesterol efflux capacity in a sub-group of the LURIC study (*Figures 1B* and *C*), in which cholesterol efflux capacity has been measured ($n = 2472$). Importantly, SDMA was a significant predictor for the HDL-associated cholesterol efflux ($P = 0.004$) while there was no association between cholesterol efflux and ADMA, corroborating the specificity of the interaction between SDMA and HDL-C. Also the first-order interaction term between SDMA and HDL-C was a significant predictor of the cholesterol efflux ($P = 0.016$ for SDMA × HDL; $P = 0.438$ for ADMA × HDL). These findings indicate that SDMA serum levels not only relate to SDMA in HDL fraction but also to HDL functionality.

To investigate the clinical relevance of these findings, we examined the effect of SDMA on the association between HDL-C concentrations and all-cause and cardiovascular mortality. For that purpose, we divided the participants of the LURIC study in those with high and low SDMA levels (above and below 90th percentile) (*Table 2*). Higher HDL-C was associated with a significant, dose-depended decrease in all-cause and cardiovascular mortality in subjects with low SDMA serum levels, even after adjustments for potential confounders such as age, gender, high-sensitive CRP, eGFR, body mass index, diabetes mellitus, smoking status, acute coronary syndrome (ACS), lipid-lowering therapy, Friesinger score, haemoglobin, and albumin. In marked contrast, in participants with SDMA concentrations exceeding the 90th percentile, the association between HDL-C and mortality was inverted and mortality was increased in subjects with higher HDL-C. To confirm these findings we additionally introduced the interaction term between HDL-C and SDMA in the Cox regression models (*Table 2*). Even after adjustment for the aforementioned variables, we found a significant interaction of HDL-C and SDMA for all-cause as well as cardiovascular mortality ($P = 0.002$ and $P = 0.008$ in the fully adjusted models). Importantly, ADMA concentrations did not show any interaction with HDL-C with respect to all-cause and cardiovascular mortality ($P = 0.428$ and $P = 0.219$ for the first-order interaction term of ADMA × HDL-C in the aforementioned fully adjusted models). These findings document the specificity of the interaction between SDMA and HDL-C, and confirm our recent experimental findings, that ADMA was absent in the HDL fractions.[25] In complementary Cox regression models, adjustment for serum amyloid A (SAA) did not change the association between HDL-C/SDMA and mortality (see Supplementary material online, *Table S2*). Moreover, in Cox regression models including HDL-C and SDMA or ADMA as continuous variables, we only found a significant interaction between HDL-C and SDMA and not ADMA (see Supplementary material online, *Table S3*). Additionally, we used hazard ratio plots to illustrate this specific interaction between HDL-C and SDMA (*Figure 2*). Notably, as shown in Supplementary material online, *Table S4*, we found that apolipoprotein A-I as well as apolipoprotein A-II were both associated with lower mortality in subjected with low SDMA. In contrast, in subjects with high SDMA, apolipoprotein A-I and apolipoprotein A-II lost their negative association with mortality or were even associated with increased risk for mortality.

To validate these findings in an independent second cohort, we performed similar analyses in the MONICA/KORA S3 study. The baseline characteristics of this cohort are presented in see Supplementary material online, *Table S5*. Notably, also in the MONICA/KORA S3 study, SDMA was an effect modifier of the association between HDL-C and mortality (*Figure 3*). Higher HDL-C levels were associated with significantly lower mortality, but only in participants with low SDMA at baseline. Similarly to our findings in the LURIC study, HDL-C completely lost its association with better outcome in participants with higher levels of SDMA.

Table 2 Association between HDL-C and all-cause and cardiovascular mortality by categories of SDMA (1st-order interaction term)

Model	Quartile of HDL-C	SDMA ≤ 0.8 μmol/L (90th percentile)		SDMA > 0.8 μmol/L (90th percentile)	
		HR (95% CI)	*P*	HR (95% CI)	*P*
All-cause mortality					
Crude	1	Reference			
	2	0.80 (0.66–0.98)	0.031	0.73 (0.46–1.16)	0.182
	3	0.71 (0.58–0.87)	0.001	1.34 (0.86–2.08)	0.198
	4	0.65 (0.53–0.81)	<0.001	1.61 (1.03–2.51)	0.036
		P = 0.014 for the interaction between HDL-C and SDMA*			
		Harrell's *C* = 0.601[a]			
Adjusted 1	1	Reference			
	2	0.80 (0.66–0.98)	0.033	0.86 (0.54–1.35)	0.502
	3	0.69 (0.56–0.85)	<0.001	1.49 (0.95–2.31)	0.080
	4	0.63 (0.50–0.78)	<0.001	1.72 (1.10–2.68)	0.017
		P = 0.016 for the interaction between HDL-C and SDMA**			
		Harrell's *C* = 0.711			
Adjusted 2	1	Reference			
	2	0.86 (0.70–1.06)	0.151	0.84 (0.53–1.33)	0.464
	3	0.78 (0.63–0.97)	0.024	1.52 (0.97–2.38)	0.066
	4	0.72 (0.57–0.91)	0.005	1.98 (1.27–3.09)	0.003
		P = 0.002 for the interaction between HDL-C and SDMA***			
		Harrell's *C* = 0.745			
Cardiovascular mortality					
Crude	1	Reference			
	2	0.71 (0.55–0.92)	0.009	0.73 (0.41–1.30)	0.286
	3	0.63 (0.48–0.82)	0.001	1.62 (0.96–2.76)	0.073
	4	0.60 (0.45–0.78)	<0.001	1.60 (0.92–2.77)	0.094
		P = 0.031 for the interaction between HDL-C and SDMA****			
		Harrell's *C* = 0.618			
Adjusted 1	1	Reference			
	2	0.71 (0.55–0.92)	0.009	0.85 (0.48–1.51)	0.571
	3	0.61 (0.47–0.79)	<0.001	1.79 (1.05–3.04)	0.032
	4	0.57 (0.43–0.75)	<0.001	1.70 (0.98–2.93)	0.062
		P = 0.031 for the interaction between HDL-C and SDMA*****			
		Harrell's *C* = 0.718			
Adjusted 2	1	Reference			
	2	0.78 (0.60–1.01)	0.059	0.81 (0.45–1.44)	0.468
	3	0.71 (0.54–0.93)	0.013	1.84 (1.08–3.14)	0.026
	4	0.69 (0.52–0.92)	0.012	1.93 (1.11–3.35)	0.020
		P = 0.008 for the interaction between HDL-C and SDMA******			
		Harrell's *C* = 0.927			

High-density lipoproteins-cholesterol was divided into quartiles: quartile 1: ≤ 31 mg/dl, quartile 2: 32–37 mg/dL, quartile 3: 38–45 mg/dL, quartile 4: ≥ 46 mg/dL.
Adjustment 1: Adjusted for age and sex.
Adjustment 2: Adjusted for age, sex, high-sensitive CRP, eGFR, body mass index, diabetes, smoking status, acute coronary syndrome, lipid-lowering therapy, Friesinger score, haemoglobin, albumin.
HR, Hazard ratio; CI, confidence interval; SDMA, symmetric dimethylarginine; HDL-C, high-density lipoproteins-cholesterol.
[a]Harrell's *C* for Cox regression model including only HDL-C: 0.545.
[b]Harrell's *C* for Cox regression model including only HDL-C: 0.557.
**P* = 0.380,
***P* = 0.306,
****P* = 0.428,
*****P* = 0.130,
******P* = 0.110, and
*******P* = 0.219 for the interaction term between HDL-C and ADMA in similar Cox regression models as shown using ADMA instead of SDMA.

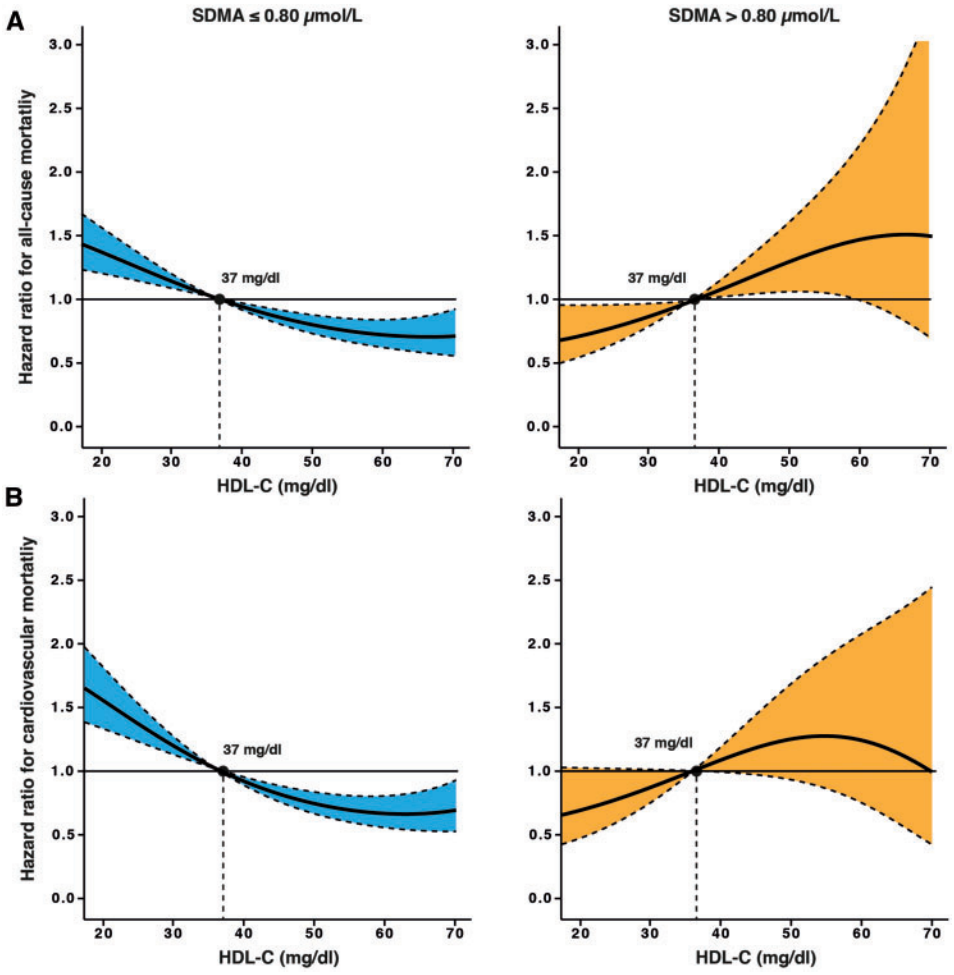

Figure 2 Hazard ratios of all-cause and cardiovascular mortality according to HDL-C levels at high and low SDMA serum levels in the LURIC study. Multivariable-adjusted hazard functions for (*A*) all-cause and (*B*) cardiovascular mortality according to HDL-C concentrations at SDMA below and above 0.8 µmol/L (90th percentile). Solid lines represent the hazard functions, dashed lines the respective 95% confidence intervals. The median of HDL-C (37 mg/dL) was chosen as reference (HR = 1.0). Adjusted for age, gender, acute coronary syndrome, Friesinger score, body-mass index, glycated haemoglobin, smoking, lipid-lowering therapy, cystatin C, high sensitivity CRP and mean systolic blood pressure. HDL-C, high-density lipoproteins-cholesterol; SDMA, symmetric dimethylarginine; LURIC, Ludwigshafen risk and cardiovascular health; HR, Hazard ratio.

Based on these findings, we developed a method, which allows the calculation of biologically effective HDL-C' based on measured HDL-C and SDMA concentrations:

$$HDL\text{-}C' = (1.869\,\mathrm{LN}(\mathrm{SDMA}) + (0.227\text{-}1.054 \cdot \mathrm{LN}(\mathrm{SDMA})) \cdot \sqrt{\mathrm{HDL\text{-}C} + 1.372})^2$$

The graph of this function is shown in *Figure 4*. Even slightly elevated SDMA sufficiently reduces 'biologically effective' HDL-C (see Supplementary material online, *Table S6*). In additional analyses, we determined the net reclassification improvement (NRI) as well as the integrated discrimination improvement (IDI) of this mathematical approach including HDL-C and SDMA over our previously described algorithm including HDL-C and SAA.[32] Notably, NRI and IDI analyses revealed a discriminative superiority of the HDL-C/SDMA model over the HDL-C/SAA model (see Supplementary methods online).

At last, we performed additional mechanistically experiments using HDL from healthy subjects supplemented with increasing concentrations of SDMA, which were comparable to those measured in HDL from CKD patients. First, we examined the effect of SDMA enriched HDL on endothelial production of ROS. Accumulation of SDMA in HDL significantly increased endothelial ROS production in a dose-dependent manner (*Figure 5A*). Furthermore, HDL from healthy subjects without SDMA reduced TNF-α stimulated endothelial VCAM-1 expression (*Figure 5B*). In contrast, HDL supplemented with SDMA failed to reduce endothelial VCAM-1 expression. Accordingly, in a monocyte-endothelial adhesion assay, we found that HDL without SDMA reduced adhesion of monocytes to TNF-α treated human

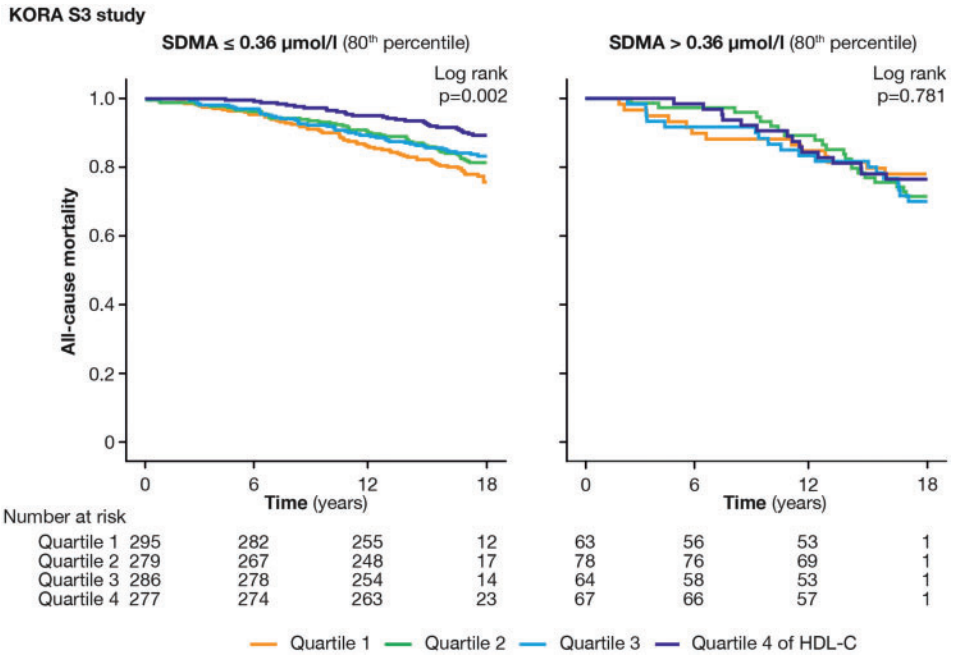

Figure 3 Survival analyses for quartiles of high-density lipoproteins-cholesterol in the MONICA/KORA S3 study. Participants were divided into two groups according to their SDMA serum levels (80th percentile). Statistical differences are calculated using Log rank test.

aortic endothelial cells (HAEC) while SDMA supplemented HDL increased the adhesion of monocytes (*Figure 5C*). These findings reveal that the accumulation of SDMA abolishes the anti-inflammatory properties of HDL. Moreover, we examined the effect of HDL on endothelial migration as a surrogate for the regenerative capacity of HDL (*Figure 5D*). HDL from healthy subjects significantly promoted the migration of HAEC in a classical scratch assay. Contrarily, SDMA-supplemented HDL lost its regenerative potential.

Discussion

Here, we not only confirm the close and specific interaction between HDL and SDMA, but for the first time demonstrate in large patient populations that SDMA is a crucial modulator of the functionality of HDL representing a novel mechanism for CVD particularly in patients with reduced renal function.

Symmetric dimethylarginine modifies the inverse relationship between HDL-C and cardiovascular events

In contrast to many other agents, which have been linked to adverse functionality of HDL only in experimental studies, the present report is the first to show the clinical relevance of these findings in two large prospective trials with long-term follow-up. We could clearly document that SDMA significantly alters the association between HDL-C and subsequent mortality. Importantly, the association between SDMA/HDL-C and mortality was even significant after adjusting for eGFR or SAA. Indeed, we have recently shown that the vasoprotective properties of HDL are increasingly lost as SDMA accumulates in HDL representing a unique mechanism leading to dysfunctional or even noxious HDL.[25] Notably, the SDMA cut-off chosen in LURIC (i.e. 90th percentile) represents a concentration, which is usually exceeded even in the earliest stages of CKD. In additional regression models, the interaction between HDL-C and SDMA as continuous variables was not dependent on a certain cut-off of SDMA. Therefore, one could speculate that even a minor reduction in kidney function as frequently observed in patients with CVD may alter the functionality of HDL. Accordingly, in previous experimental studies we found that HDL already lost its vasoprotective properties in patients with CKD in KDIGO stage G2 (eGFR 60–90 mL/min/1.73m^2).[25,26] Also in the present analyses, we could not find a specific interaction between ADMA und HDL-C, which underscores the idea that SDMA incorporation in the HDL is a CKD-specific phenomenon. Our results were consistent in two large study populations, the LURIC and the MONICA/KORA S3 study. The two studies complement each other.

While the LURIC study comprises participants undergoing coronary angiography with a high prevalence of cardiovascular risk factors, the MONICA/KORA S3 study includes participants from the general population. The consistency of the findings in both cohorts points to the general relevance of our findings. In both studies, creatinine and cystatin C were used to calculate eGFR according to the current KDIGO guidelines as state-of-the-art approach.[36] However, in participants of the LURIC study no urine samples were available to assess albuminuria. Moreover, participants of both studies were of

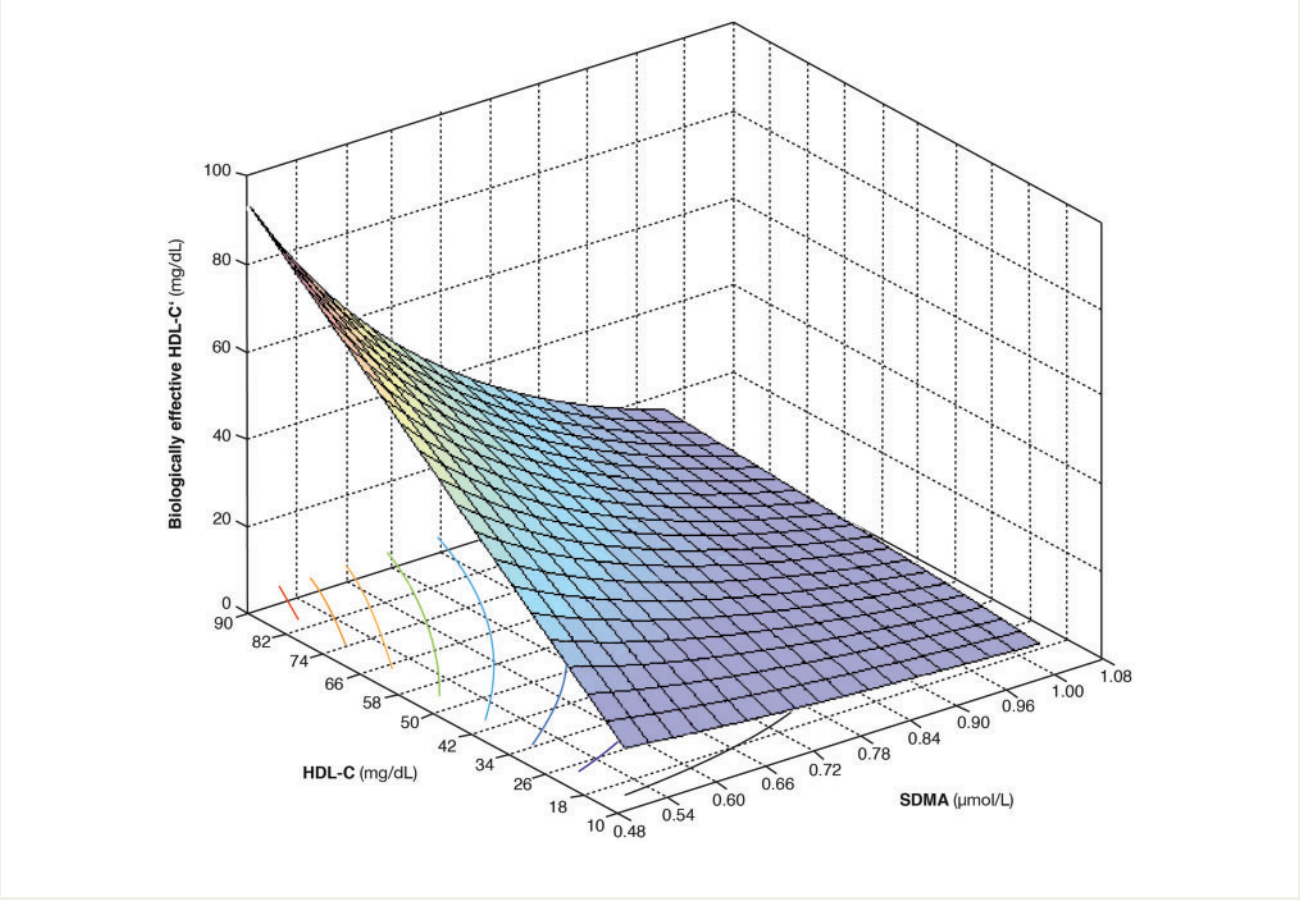

Figure 4 Graph of the function for the effective HDL-C-concentration. HDL-C'=HDL-C'(SDMA, HDL-C); isolines for HDL-C'=10,20,30, . . . ,80 mg/dL are projected onto the HDL-C-SDMA-plane; the effective HDL-C concentration HDL-C'=f(SDMA, HDL-C) (with f(x, y) = (1.869 ln(x) + (0.227 - 1.054 ln(x)) $y^{1/2}$ + 1.372)^2) is evaluated on the grid points (x_i, y_j) (i = 0, . . . ,17 and j = 0, . . . ,20), where x_i=0.48 + 0.03i, y_j=10 + 4j. The calculations and visualization have been performed by Matlab R2012a: the grid cells on the x-y plane are generated by the Matlab function *meshgrid*, the contour plot under the 3-D-shaded surface plot is created by the Matlab function *surfc*.

Caucasian ancestry, which limits the generalization of these findings to other races.

Symmetric dimethylarginine affects cellular cholesterol efflux and anti-inflammatory properties of high-density lipoproteins

The biological functions of SDMA have not been fully elucidated.[37] Whereas ADMA is well known as a direct inhibitor of eNOS,[38] SDMA is thought to be functionally inactive. Only few experimental data on the function of SDMA are available. However, it has been shown that SDMA may induce pro-inflammatory activation of mononuclear cells.[37,39] While many other modifications of HDL such as carbamylation or the incorporation of the acute phase protein SAA occur under different disease conditions such as diabetes mellitus, coronary artery disease or inflammation,[20,32] we speculate that the accumulation of SDMA in HDL may represent a CKD-specific phenomenon mostly independent of other pathological conditions. It is in line with this concept that total serum SDMA was significantly correlated with SDMA in HDL, and even more important, SDMA was a significant determinant of the HDL-associated cholesterol efflux capacity, which underscores the functional relevance of SDMA accumulation in HDL. This is additionally supported by our *in vitro* studies showing that HDL enriched with SDMA induces endothelial ROS production, VCAM-1 expression on the cell surface and subsequent endothelial mononuclear cell adhesion but inhibits endothelial cell migration. Besides these findings, we have recently shown that HDL-enriched with SDMA potently inhibits endothelial nitric oxide production.[25]

Assessment of functional high-density lipoproteins using symmetric dimethylarginine and higher HDL-cholesterol

Isolation of HDL by using density-gradient ultracentrifugation is a time-consuming and expensive procedure. It is therefore not possible to directly examine the vascular functionality of isolated HDL in clinical practice. The newly derived formula for the calculation of

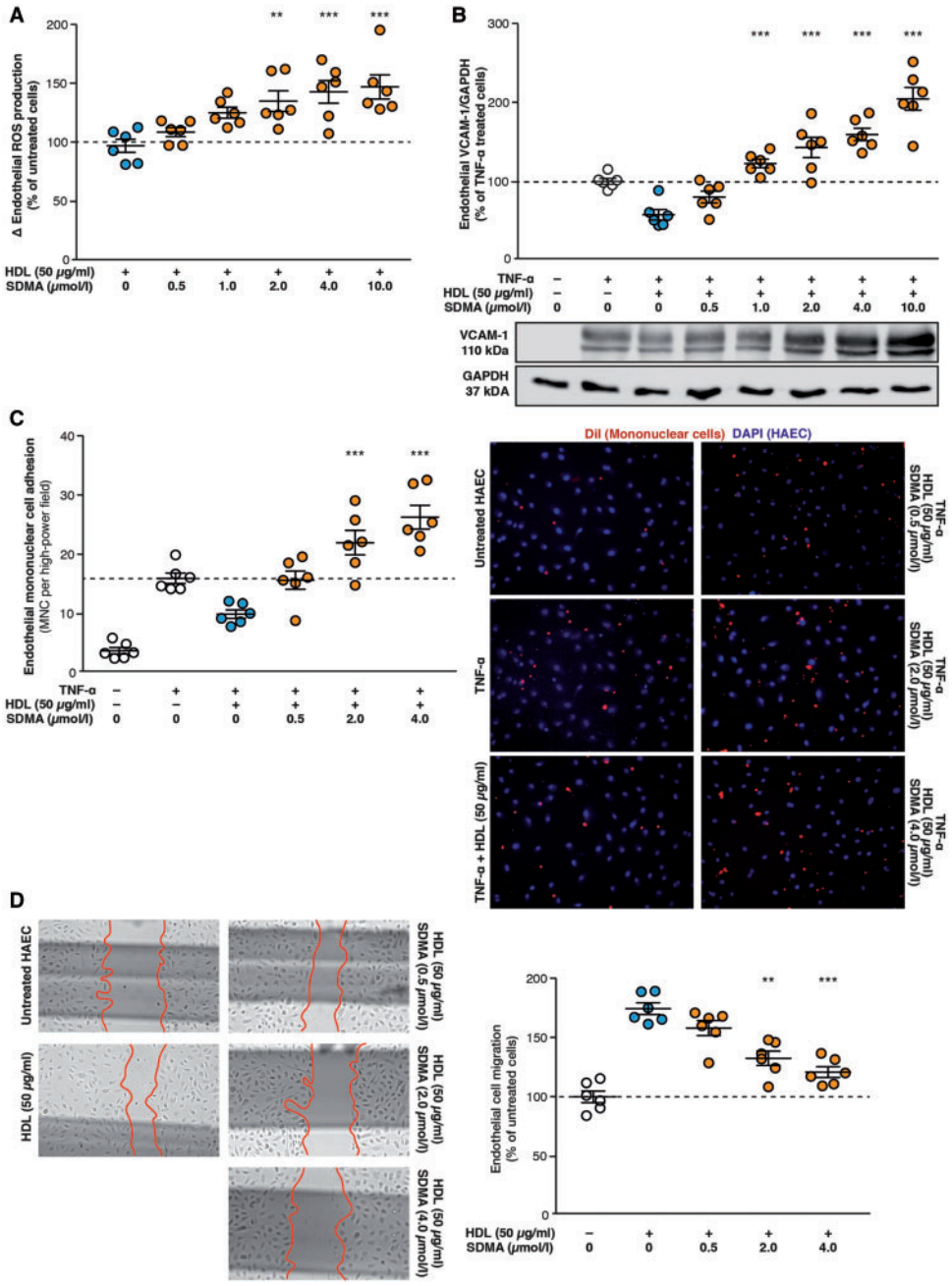

Figure 5 (*A*) Endothelial reactive oxygen species production in human aortic endothelial cells (HAEC) as determined by using ESR spectroscopy after incubation with HDL (50 μg/mL protein, 1 h) supplemented with increasing concentrations of SDMA. (*B*) Endothelial VCAM-1 expression in HAEC pre-incubated with HDL (50 μg/mL, 1 h) supplemented with SDMA and then stimulated for 4 h with TNF-α (0.1 ng/mL). (*C*) Adhesion of mononuclear cells (Dil, Red) to HAEC (DAPI, Blue) pre-incubated with HDL (50 μg/mL, 1 h) supplemented with SDMA and then stimulated for 4 h with TNF-α (0.1 ng/mL). Numbers of cells per high-power field are given. (*D*) Migration of HAEC in scratch assay incubated with HDL (50 μg/mL, 16 h) supplemented with increasing concentrations of SDMA ($n = 6$ per group).

'biologically effective', i.e. 'protective' HDL-C' represents an important tool to estimate the functionality of HDL superseding time-consuming isolations of HDL as well as expensive laboratory procedures to determine the vascular function of HDL. Our findings suggest that the relationship between HDL-C and SDMA represents a useful surrogate of the vascular functionality of HDL. In addition, the equation outperforms our recently developed algorithm including HDL-C and SAA.[32] Notably, the current mathematical algorithm is only

validated for SDMA concentrations between 0.48 and 1.20 μmol/L. Symmetric dimethylarginine concentrations above 1.20 μmol/L indicate a more severely reduced eGFR. Accordingly, we have previously shown that in patients with moderate to severe CKD, HDL not only looses its vasoprotective properties but turns into noxious particle promoting vascular damage.[25,26] Therefore, it is not possible to calculate biologically effective HDL in patients with more advanced CKD.

Symmetric dimethylarginine measurements were performed using different methods in LURIC and MONICA/KORA S3, HPLC and LC-MS/MS, respectively. Absolute levels of dimethylarginines may differ according to the assay.[40] In MONICA/KORA S3, SDMA was lower than in the LURIC study, which may be due to (i) different measurement methods and/or (ii) different study populations. Therefore, our study cannot reveal a definite SDMA cut-off, at which HDL functionality is impaired and our algorithm to determine functional HDL is provisionally for the HPLC method used in LURIC before a standardization of SDMA methods has been achieved.

In summary, SDMA modulates the association between HDL-C and mortality in two study populations using two distinct methods to measure SDMA as a specific HDL-modulating agent. Our findings establish SDMA as an essential effector of HDL function and as clinically relevant cause for CKD-associated vascular disease. Cholesterol efflux data and *in vitro* experiments provide insights into the biochemical mechanism underlying our clinical observations. A simple approach is suggested to estimate 'biologically effective HDLC', which might represent a useful novel clinical tool for use in humans, especially in patients with impaired renal function.

Supplementary material

Supplementary material is available at *European Heart Journal* online.

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