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Asymmetric Dimethylarginine, Smoking, and Risk of Coronary Heart Disease in Apparently Healthy Men: Prospective Analysis from the Population-Based Monitoring of Trends and Determinants in Cardiovascular Disease/Kooperative Gesundheitsforschung in der Region Augsburg Study and Experimental Data

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Background: **An increased plasma concentration of the endogenous nitric oxide synthase inhibitor asymmetric dimethylarginine (ADMA) predicts adverse clinical outcome in patients with coronary heart disease. We investigated the association between plasma concentrations of ADMA and risk in initially healthy smoking and nonsmoking men.**

Methods: **Participants for this nested case-control study came from the population-based Monitoring of Trends and Determinants in Cardiovascular Disease/Kooperative Gesundheitsforschung in der Region Augsburg study. ADMA was measured by liquid chromatography–tandem mass spectrometry in 88 men with incident coronary events (fatal and nonfatal myocardial infarction and sudden cardiac death) and 254 age-matched controls, with a median (interquartile range) follow-up of 6.2 (3.3–7.9) years.**

Results: **After adjustment for potential confounders, the relative risk for a future coronary event was 2.00 [95% confidence interval (CI) 1.27–3.16; $P = 0.003$] for smokers compared with nonsmokers and 1.35 (95% CI 0.78–2.33; $P = 0.282$) for the top vs the bottom tertile of the ADMA distribution. In cases and controls, lower ADMA plasma concentrations were observed in smokers. Analysis of ADMA-associated risk in smokers and nonsmokers separately revealed substantial differences: the adjusted relative risk for future coronary events (top vs bottom tertile of the ADMA distribution) was 0.48 (95% CI 0.16–1.46; $P = 0.198$) in smokers and 2.40 (95% CI 1.14–5.08; $P = 0.021$) in nonsmokers. Exposure of human endothelium-derived EAhy 926 cells to tobacco smoke enhanced expression of the ADMA metabolizing enzyme dimethylarginine dimethylaminohydrolase 2 and reduced ADMA concentration.**

Conclusions: **In apparently healthy men, increased ADMA predicts the risk for coronary events in nonsmokers, but not in smokers. This may be explained in part by an alteration of ADMA metabolism by tobacco smoke.**

Accumulating evidence is linking asymmetric dimethylarginine (ADMA),[4] an endogenous inhibitor of all major isoforms of nitric oxide synthase (NOS), to human disease

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[4] Nonstandard abbreviations: ADMA, asymmetric dimethylarginine; NOS, nitric oxide synthase; CHD, coronary heart disease; MONICA, Monitoring of Trends and Determinants in Cardiovascular Disease; KORA, Kooperative Gesundheitsforschung in der Region Augsburg; BMI, body mass index; CSC, cigarette smoke condensate; DDAH, dimethylarginine dimethylaminohydrolase; TC, total serum cholesterol; HDL-C, HDL cholesterol; CRP, C-reactive protein; LDH, lactate dehydrogenase; GFR, glomerular filtration rate; MDRD, Modification of Diet in Renal Disease; HR, hazard ratio; CI, confidence interval.

(1, 2). Increased ADMA plasma concentrations are found in various clinical settings ranging from renal failure and liver failure to atherosclerosis, hypertension, and impaired glucose tolerance *(3-6)*. Moreover, increase of ADMA has been identified as an independent risk factor for progression of atherosclerosis, cardiovascular death, and total mortality in patients with coronary heart disease (CHD) *(7-9)* and renal failure *(10, 11)* and in critically ill patients *(12)*. In the only large prospective study that included patients with and without CHD, all smokers (29% of patients) had been excluded from the main statistical analysis, and a significantly increased risk for those in the highest tertile of plasma ADMA distribution was mainly confined to men with a previous history of CHD *(7)*. Data are still lacking that clearly demonstrate that an increase of ADMA is also associated with death and cardiovascular events in patients without CHD or organ failure. Moreover, the underlying mechanisms responsible for the confounding effect of smoking on ADMA-associated risk remain to be elucidated. We therefore conducted a nested case-control study to assess the potential relevance of ADMA as a risk factor for coronary events in smoking and nonsmoking men without a history of CHD.

Materials and Methods

STUDY DESIGN AND PARTICIPANTS

We set up this study as a prospective case-control study within the population-based Monitoring of Trends and Determinants in Cardiovascular Disease (MONICA) Augsburg surveys, conducted in 1989/1990 (S2) and 1994/1995 (S3). The MONICA Augsburg project was part of the multinational WHO MONICA project *(13, 14)*, for which men and women from a general population of more than 280 000 inhabitants of a mixed urban/rural area were randomly invited to participate (response rates for participation in the S2 and S3 surveys were 76.9% and 74.9%, respectively). The study was approved by the local ethics committee, and all participants provided written informed consent.

Altogether, 9796 men and women ages 25 to 74 years participated in the 2 independent cross-sectional surveys (S2, 4940 participants; S3, 4856 participants). In the framework of the Kooperative Gesundheitsforschung in der Region Augsburg (KORA), vital statistics were assessed for all sampled persons in the 2 survey populations. Patients with incident acute coronary events before the 75th year of age were identified through the population-based MONICA/KORA coronary event registry *(15)*. The median (interquartile range) follow-up time of the study population was 6.2 (3.3–7.9) years. Eighty-nine men without prevalent CHD or diabetes mellitus at baseline developed an incident acute coronary event (S2, 78 cases; S3, 11 cases). For each case, we randomly selected 3 age- and survey-matched control individuals without an incident acute coronary event during follow-up from the 2 survey populations, resulting in a study sample of 356 participants (89 cases, 267 controls). We decided not to include women because of their very low event rate. We excluded from the analysis 14 men (1 case, 13 controls) with missing data for ADMA or any of the other considered variables. Therefore, the study population of the present report is based on 88 cases and 254 event-free controls ages 35 to 74 years.

The outcome variable was a combination of incident fatal or nonfatal acute myocardial infarction and sudden cardiac death. According to the MONICA manual *(14)*, the diagnosis of a major nonfatal myocardial infarction was based on acute symptoms, cardiac enzymes, and typical electrocardiogram changes. Deaths from cardiovascular causes were validated by autopsy reports, death certificates, chart review, and information from the last treating physician.

SURVEY METHODS

All participants completed a standardized questionnaire, including medical history, lifestyle, and drug intake. Blood pressure, body height (m), body weight (kg), body mass index (BMI; kg/m^2), smoking habits, and alcohol consumption (g/day) were determined as described *(16)*. We assessed leisure-time physical activity on a 4-level graded scale for winter and summer (none, <1, 1–2, and >2 h/week) and calculated the number of years of education from the highest level of formal education completed *(17)*. Actual hypertension was defined as systolic blood pressure $\geq$140 mm Hg and/or diastolic blood pressure $\geq$90 mm Hg, being aware of having hypertension, or taking antihypertensive medication.

CELL CULTURE EXPERIMENTS

We maintained human endothelial cells (EAhy 926 cells, a hybrid human cell line derived from fusion of human umbilical vein endothelial cells and A549 carcinoma cells, a kind gift of Dr. Edgell (University of North Carolina, Chapel Hill, NC) *(18)* in DMEM at 37 °C in a humidified atmosphere containing 50 mL CO_2/L. The cigarette smoke condensate (CSC) used in the cell culture experiments was prepared from the University of Kentucky reference cigarette 2R4F. We collected the particulate phase of smoke on a Cambridge filter pad by use of a smoking machine under standard conditions prescribed by ISO4387:2000. The smoke particulate matter was dissolved in dimethyl sulfoxide at 10 g/L and stored frozen at -80 °C in separate vials. On the day of the experiment, each vial of CSC solution was opened and diluted in serum-free cell culture medium to desired concentration. Nicotine concentrations observed at CSC concentrations of 1 and 10 mg/L matched the concentration interval found in plasma of smokers. We assessed ADMA liberation, RNA expression, and cytotoxicity in 6-well plates after treatment of cells for 48 h with CSC. Control cells were treated with medium containing an equivalent amount of dimethyl sulfoxide.

TaqMan real-time quantitative reverse transcription-PCR analysis

Total RNA was extracted from EAhy 296 cells by RNAzol (Wak-Chemie) and reverse-transcribed (Superscript II, Invitrogen) by use of random hexamers. We quantified mRNA expression of dimethylarginine dimethylaminohydrolase 1 (DDAH1) and DDAH2 by use of the Applied Biosystems ABI Prism 7900 HT system (TaqMan). We carried out TaqMan reactions in 384-well plates according to the manufacturer's instructions (Applied Biosystems) using premade probes for DDAH1 (Probe Hs00201707_m1 generating an amplicon of 77 bp from the NM_012137.2 transcript) and DDHA2 (probe Hs00203889_m1 generating an amplicon of 85 bp from the NM_013974.1 transcript) and glyceraldehyde-3-phosphate dehydrogenase as an endogenous control (probe Hs99999905_m1 generating an amplicon of 122 bp from the M33197.1 transcript). We performed relative quantification of gene expression using the ΔΔCT method as described in the user guide for the ABI Prism 7900 HT system.

Laboratory procedures

We collected a nonfasting venous blood sample from all participants in a supine resting position. We measured total serum cholesterol (TC) and HDL cholesterol (HDL-C) by routine enzymatic methods. Samples for measurement of C-reactive protein (CRP) and ADMA were stored at −70 °C until analysis. We measured serum CRP concentrations with a high-sensitivity immunoradiometric assay (range, 0.05 to 10 mg/L) as described *(19)*. In cell culture supernatants, we measured ADMA and L-arginine by ultrasensitive liquid chromatography–tandem mass spectrometry as previously validated and described in detail *(20)*. We measured plasma concentrations of ADMA by use of a commercial ELISA reagent set (DLD) *(21)*. The lower limit of detection for the ADMA ELISA was <0.05 μmol/L; intra- and interassay CVs were 4.5% and 8.3%, respectively. Cross-reactivities with SDMA and L-arginine were 1.2% and <0.02%, respectively. The correlation coefficient of the plasma ADMA values obtained by ELISA and liquid chromatography–tandem mass spectrometry (n = 29) was 0.984.

We measured oxidized LDL by use of a competitive ELISA (Mercodia). We measured lactate dehydrogenase (LDH) by use of a commercial reagent set (cytotoxicity detection reagent set; Roche Diagnostics) and protein concentration by use of the Bradford protein assay (Bio-Rad). We estimated glomerular filtration rate (GFR) according to the abbreviated Modification of Diet in Renal Disease (MDRD) formula *(22)*.

All analyses were run in a blinded fashion.

Statistical analysis

We computed means or proportions for baseline demographic and clinical characteristics for men with and without an incident coronary event. The distribution of CRP concentration was markedly skewed, and we therefore calculated the geometric mean (geometric SD) from the log-transformed data. We tested differences in mean values of continuous variables for statistical significance by *t*-test and differences in proportions by χ^2 test. We assessed associations among continuous variables by Pearson correlation coefficient *r*.

We used Cox proportional hazards analysis to assess the independent risk for the occurrence of a 1st coronary event. Study participants were grouped into tertiles of plasma ADMA concentrations, and the risk was calculated relative to the bottom tertile. Results are presented as hazard ratios (HRs) together with their 95% confidence interval (CI). First, crude HRs were calculated (model 1). Results were then adjusted for age (continuous) and survey (S2 or S3; model 2) and for age (continuous), survey (S2, S3), education years (<12, ≥12 years), smoking status (yes, no), alcohol consumption (0 g/d, 1–39.9 g/d, ≥40 g/d), obesity (BMI <30.0 kg/m^2, ≥BMI≥30.0 kg/m^2), physical activity (inactive, active), actual hypertension (no, yes), TC/HDL-C ratio (continuous), and GFR (continuous; model 3). To further evaluate the interaction of ADMA and smoking status with regard to incident coronary events, we calculated adjusted HRs according to smoking status and tertile of ADMA, with never-smoking and low ADMA tertile as reference. For a test of trend, we coded ADMA tertiles with their median values and repeated the Cox regression described above. Moreover, to test for possible modifications of the ADMA effect on a coronary event by risk factors, we included interaction terms of ADMA tertiles and the parameter under concern in the multivariate Cox regression models. Cell culture data were compared by ANOVA followed by Dunnett multiple comparison test.

All significance tests were 2-tailed, and probability values <0.05 were considered statistically significant. All analyses were performed with the Statistical Analysis System (version 8.2, SAS Institute) and Prism4 (GraphPad Software).

Results

Baseline characteristics

Baseline characteristics of cases and controls are shown in Table 1. The median (interquartile range) time to a 1st coronary event was 2.9 (1.2–5.1) years. Men who experienced an event were more frequently smokers or had hypertension compared with controls. Likewise, significantly higher concentrations of CRP, TC (and TC/HDL-C ratio), and oxidized LDL were measured in cases compared with controls, whereas the difference in BMI reached only borderline significance. Cases and controls did not differ significantly in age (matching variable), educational level, physical activity, alcohol intake, diastolic blood pressure, or HDL-C. Mean (SD) plasma ADMA concentrations in participants who experienced an event and in controls were also similar: 0.80 (0.22) and 0.79 (0.21) μmol/L, $P = 0.72$.

Table 1. Demographic and clinical characteristics of men with (cases) and without (controls) incident coronary event.[a]

Characteristics	Cases	Controls	*P* value
n	88	254	
Age, years	61.1 (8.6)	61.2 (8.4)	0.898
Education, <12 years[b]	76.1	75.2	0.860
Body mass index, kg/m^2	28.6 (4.0)	27.7 (3.7)	0.058
Actual hypertension[b]	76.1	56.7	0.001
Systolic blood pressure, mmHg	146.0 (22.3)	138.8 (18.2)	0.007
Diastolic blood pressure, mmHg	82.6 (13.2)	81.9 (10.7)	0.671
Physical activity[b]	31.8	29.5	0.686
Smoking status[b]			0.013
Never smoker	21.6	31.9	
Former smoker	42.0	46.9	
Current smoker	36.4	21.3	
Alcohol intake[b]			0.746
0 g/day	18.2	16.9	
0.1–39.9 g/day	48.9	53.5	
≥40 g/day	33.0	29.5	
TC, mmol/L	6.6 (1.1)	6.3 (1.1)	0.014
HDL-C, mmol/L	1.3 (0.4)	1.3 (0.4)	0.262
TC/HDL-C	5.5 (1.6)	5.0 (1.8)	0.023
Oxidized LDL, U/L	109.8 (32.1)	93.2 (28.0)	<0.001
CRP, mg/L[c]	2.8 (3.2)	1.7 (3.0)	0.001
Creatinine, μmol/L	97.2 (26.5)	97.2 (17.7)	0.198
GFR, mL/min[d]	75.5 (18.7)	77.4 (17.3)	0.368
ADMA, μmol/L	0.80 (0.22)	0.79 (0.21)	0.721

[a] Data are arithmetic mean (SD), *P* value from *t*-test, unless noted otherwise.
[b] %, *P* value from χ^2 test.
[c] Geometric mean (geometric SD), *P* value from *t*-test after log transformation (n = 316, 235 controls, 81 cases).
[d] Calculated according to the abbreviated MDRD formula.

associations and correlations between adma and cardiovascular risk factors

Correlations between ADMA and cardiovascular risk factors are shown in Table 2. ADMA and age were positively correlated ($r = 0.28$, $P < 0.001$ in controls and $r = 0.41$, $P < 0.001$ in cases), whereas there was an inverse correlation of ADMA and GFR ($r = -0.25$, $P < 0.001$ in controls and $r = -0.326$, $P = 0.002$ in cases). We observed no statistically significant correlations of ADMA with BMI or with systolic or diastolic blood pressure, TC, HDL-C, oxidized LDL, or CRP. In both groups (cases and controls), differences in ADMA concentrations between smokers and nonsmokers were observed: mean (SD) ADMA concentrations among cases were 0.69 (0.17) and 0.87 (0.23), respectively ($P < 0.001$), and among controls, 0.74 (0.20) and 0.80 (0.22), respectively ($P = 0.067$).

relative risks for coronary events associated with increase of adma in relation to smoking status

To assess the risk of an incident coronary event according to baseline concentrations of ADMA, we calculated Cox proportional hazard models (Table 3). For this purpose, ADMA concentrations were categorized into tertiles (0.36–0.70, 0.71–0.85, and 0.86–2.30 μmol/L). Median ADMA plasma concentrations in the low, intermediate, and high ADMA tertiles were 0.61, 0.77, and 0.97 μmol/L, respectively.

When all 342 men were included, irrespective of smoking status, the trend toward a higher HR for an incident coronary event in the intermediate and the top tertile vs the bottom tertile of the ADMA distribution did not reach statistical significance in any of the Cox regression models (Table 3). In the fully adjusted model *(3)* the HR for the top ADMA tertile was 1.35 (95% CI 0.78–2.33; $P = 0.282$). In the same model, the relative risk of a future coronary event was 2.00 (95% CI 1.27–3.16; $P = 0.003$) for smokers compared with nonsmokers. By contrast, analysis of ADMA-associated risk for smokers and nonsmokers separately revealed striking differences (Table 3). The adjusted relative risk comparing the top tertile to the bottom tertile of the ADMA distribution was 0.48 (95% CI 0.16–1.46; $P = 0.198$) for smokers, but it was 2.40 (95% CI 1.14–5.08; $P = 0.021$) for nonsmokers. Smoking was the only risk factor with a significant interaction with ADMA values ($P = 0.031$). In further analysis, adjusting for CRP (n = 316), the HRs were similar, with HRs (95% CIs) of 0.48 (0.14–1.66) for smokers and 2.35 (1.10–5.03) for nonsmokers.

To explore the interaction of ADMA and smoking in more detail, we assessed HRs after grouping the participants according to smoking status and tertile of ADMA

Table 2. Correlation and association between ADMA and cardiovascular risk factors in cases and controls.[a]

	ADMA			
Risk factor	**Cases**	***P* value**	**Controls**	***P* value**
Age	0.414	<0.001	0.284	<0.001
BMI	−0.051	0.640	−0.005	0.933
Systolic blood pressure	−0.065	0.546	0.040	0.523
Diastolic blood pressure	−0.112	0.297	−0.079	0.209
TC	−0.041	0.702	−0.058	0.362
HDL-C	−0.021	0.844	−0.093	0.142
TC/HDL-C	−0.070	0.517	0.038	0.551
Creatinine	0.198	0.002	0.229	0.032
GFR[b]	−0.326	0.002	−0.250	<0.001
Oxidized LDL	0.054	0.395	−0.078	0.486
Log CRP[c]	0.056	0.621	0.047	0.473
Actual hypertension[d]		0.606		0.944
No	0.78 (0.22)		0.79 (0.21)	
Yes	0.81 (0.23)		0.79 (0.22)	
Physical activity[d]		0.816		0.848
No	0.81 (0.24)		0.79 (0.19)	
Yes	0.79 (0.19)		0.80 (0.27)	
Smoker[d]		0.001		0.165
Never	0.88 (0.24)		0.80 (0.20)	
Former	0.85 (0.22)		0.81 (0.23)	
Current	0.69 (0.17)		0.74 (0.20)	

[a] Data are Pearson correlation coefficient *r* unless noted otherwise.
[b] Calculated according to the abbreviated MDRD formula.
[c] 81 cases, 235 controls.
[d] Mean (SD).

(Fig. 1). Using as reference nonsmoking men with plasma ADMA in the lowest tertile (n = 77), the complex interaction of smoking and ADMA with respect to coronary risk became even more evident. In accordance with the initial analysis in nonsmoking men, the risk of suffering a coronary event increased with tertiles of ADMA [HR 1.63 (0.77–3.46) and 2.37 (1.14–4.95), respectively, for men in the 2nd (n = 88) and 3rd (n = 91) ADMA tertiles]. By contrast, the coronary risk in the middle and top ADMA tertiles did not further increase in current smokers [HR 2.25 (0.87–7.79) in the 2nd ADMA tertile (n = 24) and HR 2.36 (0.81–6.86) in the 3rd ADMA tertile (n = 17)]. Smoking status was associated with the highest risk in patients with low ADMA concentrations (n = 45) [HR 4.49 (2.08–9.68)].

effects of tobacco smoke on adma liberation and ddah expression in EAhy 926 cells

Incubation of EAhy 926 cells for 48 h with 1.0 and 10.0 mg/L CSC resulted in declines of the ADMA concentration in the cell culture medium (normalized to cellular protein) by 28.2% and 24.8%, respectively (Fig. 2A), whereas very low concentrations of CSC had no effect. The ADMA/L-arginine ratio declined with increasing concentrations (1.0 and 10.0 mg/L) of CSC, by −18.2% and −22.0%, respectively (both $P < 0.05$).

Expression of DDAH2 mRNA was augmented by 87.6% after 48 h of incubation with 10.0 mg/L tobacco smoke concentrate, whereas changes in expression did not reach statistical significance at lower concentrations (Fig. 2B). Expression of DDAH1 was not significantly altered (all $P > 0.05$). None of the concentrations of CSC was associated with significant increases in LDH activity (as indicator of cytotoxicity; Fig. 2C).

Discussion

The major finding of this prospective population-based study of 342 men (88 incident cases and 254 age-matched controls) without a history of CHD or diabetes mellitus at baseline is that increase of ADMA is an independent risk factor for future fatal and nonfatal coronary events in nonsmoking men but not in smoking men. With respect to ADMA and smoking, our findings point to a significant interaction of smoking and ADMA-associated cardiovascular risk. Furthermore, our cell culture experiments indicate that CSC may enhance degradation of ADMA by upregulation of DDAH2.

smoking and adma

So far, the effect of smoking on ADMA has been discussed quite controversially *(23)*. In a previous study in elderly men with a high cardiovascular risk profile, significantly lower plasma ADMA concentrations were no longer statistically significant after correction for baseline variables and morbidity *(24)*. Another recent study by Schnabel et al. *(9)* found higher ADMA concentrations in

Table 3. Relative risk for the incidence of a coronary event estimated by HR with 95% CIs.

Tertile of ADMA Model	n, Cases/controls	Low ADMA, 0.36–0.70 μmol/L Reference value	Intermediate ADMA, 0.71–0.85 μmol/L	*P*, intermediate vs low ADMA	High ADMA, 0.86–2.30 μmol/L	*P*, high vs low ADMA	*P* for trend
All participants	88/254						
Unadjusted		1	1.09 (0.65–1.83)	0.742	1.27 (0.77–2.11)	0.356	0.354
Adjusted for age and survey		1	1.02 (0.60–1.73)	0.953	1.20 (0.70–2.04)	0.505	0.488
Multivariable adjustment[a]		1	0.97 (0.56–1.67)	0.901	1.35 (0.78–2.33)	0.282	0.262
Current smokers	32/54						
Unadjusted		1	0.76 (0.33–1.73)	0.506	0.72 (0.27–1.92)	0.505	0.439
Adjusted for age and survey		1	0.75 (0.32–1.78)	0.515	0.71 (0.25–2.00)	0.517	0.468
Multivariable adjustment[b]		1	0.39 (0.15–1.04)	0.060	0.48 (0.16–1.46)	0.198	0.150
Former smokers	37/119						
Unadjusted		1	1.12 (0.46–2.69)	0.808	1.74 (0.78–3.91)	0.179	0.148
Adjusted for age and survey		1	0.98 (0.40–2.39)	0.957	1.51 (0.66–3.48)	0.332	0.261
Multivariable adjustment[b]		1	1.01 (0.40–2.57)	0.986	1.55 (0.66–3.65)	0.315	0.263
Never smokers	19/81						
Unadjusted		1	5.05 (1.09–23.44)	0.039	4.43 (0.94–20.86)	0.060	0.086
Adjusted for age and survey		1	4.95 (1.04–23.57)	0.045	4.38 (0.89–21.61)	0.070	0.111
Multivariable adjustment[b]		1	3.96 (0.79–19.93)	0.096	7.06 (1.30–38.40)	0.024	0.019
Never smokers and former smokers	56/200						
Unadjusted		1	1.83 (0.88–3.82)	0.109	2.28 (1.12–4.64)	0.023	0.025
Adjusted for age and survey		1	1.66 (0.79–3.52)	0.184	2.07 (0.99–4.30)	0.053	0.058
Multivariable adjustment[b]		1	1.77 (0.83–3.78)	0.138	2.40 (1.14–5.08)	0.021	0.022

[a] HRs for Cox regression models adjusting for age, survey, education level, alcohol consumption, obesity, physical activity, hypertension, TC/HDL-C ratio, GFR, and smoking status.

[b] HRs for Cox regression models adjusted for age, survey, education level, alcohol consumption, obesity, physical activity, hypertension, TC/HDL-C ratio, and GFR.

smoking compared with nonsmoking men and women in a population of patients with preexisting CHD. The most plausible explanation for apparent discrepancies regarding the effects of smoking on ADMA reported by different investigators is that ADMA plasma concentrations are regulated by multiple factors and that in health and disease the relative contributions of these mechanisms to ADMA concentrations may be quite different *(1, 25)*.

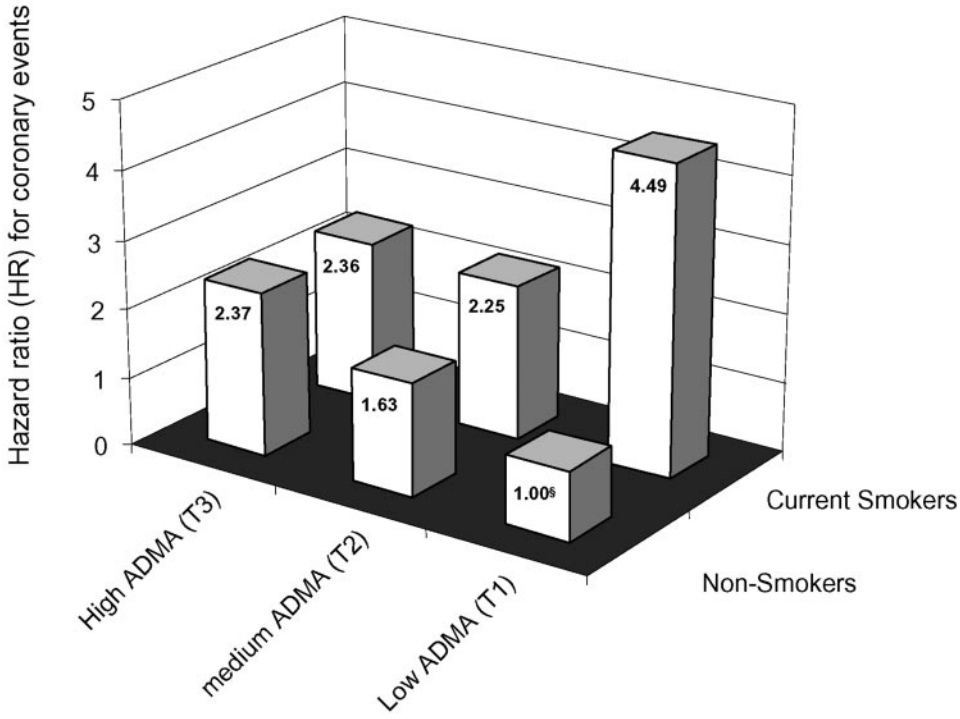

Fig. 1. HRs for a 1st coronary event in men categorized in tertiles of plasma ADMA concentration and smoking status.

Cox regression model adjusted for age, survey, education level, alcohol consumption, obesity, physical activity, hypertension, and TC/HDL-C ratio. §, reference (low ADMA tertile and nonsmokers).

Most data available today suggest that degradation of ADMA by DDAH, rather than asymmetrical methylation of L-arginine residues and proteolysis, plays the key role in the regulation of ADMA concentrations *(1, 25)*. Both isoforms of DDAH have been shown to be sensitive to oxidative stress *(26)*, and ADMA concentrations tend to rise under conditions of oxidative stress *(27)*. Hence, differences in systemic oxidative stress in patients with and without underlying cardiovascular disease may explain some of the apparent discrepancies.

A previous analysis of the MONICA Augsburg 1994/1995 cohort found a strong association between smoking and various markers of systemic inflammation in men *(28)*. Unlike oxidative stress, inflammation is associated with lower ADMA concentrations *(29)*. Although in our study there was no formal statistical interaction between ADMA and CRP, smoking-induced inflammation may have contributed to lower ADMA concentrations.

This study was the first to investigate a complete (lipid and water soluble) extract of cigarette smoke; previous studies reporting divergent results on ADMA and DDAH expression used only the water-soluble fraction of cigarette smoke *(30)* or nicotine at cytotoxic levels *(31)* or investigated ADMA concentrations in regenerated endothelial cells from carotid arteries obtained 6 weeks after endothelial removal in rabbits chronically exposed to nicotine *(32)*.

A further explanation for divergent observations may be that CSCs from different cigarette brands were found to differ in their effect on the expression of a subset of >1000 genes *(33)*.

SMOKING, ADMA, AND CORONARY RISK

This is the first prospective study investigating the association of ADMA and cardiovascular risk in a nested case-control design in a large cohort of primarily healthy participants. All previous studies on ADMA involved patients with preexisting CHD *(6, 9)* or organ failure *(11, 12, 34)* or mixed cohorts *(7)*. In the whole cohort on which the present study was based (n = 3022), the incidences of fatal and nonfatal coronary events per 100 000 persons for current smokers, former smokers, and men who never smoked were 723, 578, and 399, respectively. With this in mind, it is striking that in the present study we observed little or no additive effect of smoking on risks in the middle and top ADMA tertiles (Fig. 1). Even more surprising is the observation that in men with low ADMA values, smoking was associated with the highest HR. Based on our in vitro data (which suggest that cigarette smoke may enhance metabolism of ADMA) a simple explanation for this apparent paradox may be that with respect to ADMA-associated risk, smoking may present a classical confounding factor. High cigarette consumption may lower ADMA concentrations while contributing itself to a higher incidence of cardiovascular events via multiple other mechanisms.

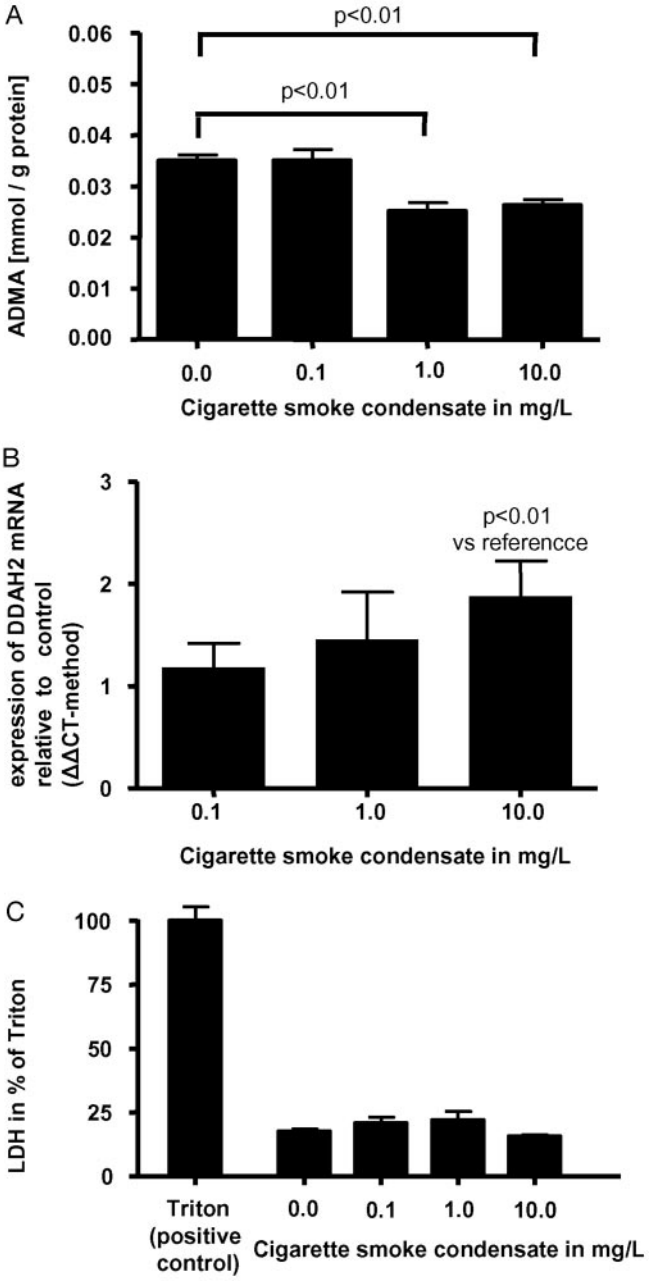

Fig. 2. EAhy 926 cells were cultured for 48 h with different concentrations (0.0, 0.1, 1.0, and 10.0 mg/L) of cigarette smoke concentrate.

(*A*), Concentrations of ADMA in conditioned medium expressed as mmol/g total cellular protein. CSC significantly reduced accumulation of ADMA in the conditioned medium. Data are mean and SE of 12 different experiments and expressed relative to control. A significant reduction of ADMA was observed with 1.0 and 10.0 mg/L cigarette smoke concentrate, $P < 0.01$ and $P < 0.001$, respectively. (*B*), Relative expression of DDAH2 mRNA in EAhy 926 cells quantified by TaqMan reverse transcription PCR using glyceraldehyde-3-phosphate dehydrogenase as internal standard. Data are mean and SE of expression relative to control (ΔΔCT). Data are mean and SE of 12 different experiments. A significant increase of DDAH2 expression ($P = 0.025$ vs reference) was observed with 10.0 mg/L CSC. (*C*), Cytotoxicity of CSC was assessed as LDH liberation from cultured cells in % of LDH liberation induced by Triton X-100 (positive control). Data are mean and SE of 6 different experiments. No significant differences were detected.

Upregulation of DDAH expression should not raise too much enthusiasm in smokers, though. Smoking clearly is

associated with a higher risk for cancer, and promotion of NO-mediated angiogenesis could have catastrophic effects when occurring in tumors. Indeed, recent studies suggest that DDAH activity and expression are increased in human tumors *(35)* and may enhance tumor growth *(36)*. Thus, induction of DDAH expression may be an additional mechanism linking smoking and cancer.

ADMA and other risk factors

Of all risk markers evaluated (including CRP and oxidized LDL), we found a positive correlation of ADMA concentrations only with age and a negative correlation only with GFR. Conflicting results regarding the correlation or association of ADMA and other risk markers have been previously noted *(1)* and have been attributed at least in part to differences in concomitant diseases and risk factors. In contrast to most previous studies, we investigated a rather healthy, unselected, population-based study cohort. Negative findings in our population certainly do not preclude significant correlations in higher-risk populations with more extreme deviations from the normal range of BMI or blood pressure. In this respect, it is of interest to note that in healthy men an infusion of ADMA (leading to a more than 20-fold increase of plasma ADMA concentration) resulted in an increase of the mean arterial blood pressure by as little as 4.5 mm Hg *(37)*.

Strengths and limitations of the study

The major strengths of the present study are its population-based design and the length of follow-up. We took all incident cases from 2 random samples of the general population and age-matched controls from the same source. Also, we analyzed only the hard endpoints fatal and nonfatal myocardial infarction and sudden cardiac death. Nonetheless, there are limitations of the present study that need to be considered. Whereas the sample size in this event-based nested case-control design was clearly adequate to detect or exclude clinically meaningful differences in outcome for the primary comparison, sample size for the subgroup analysis of smokers and nonsmokers was small. This resulted in relatively wide CIs, indicating limited power of the subgroup analyses, especially with regard to negative findings and absolute effect sizes.

In conclusion, in an apparently healthy population with a low to moderate cardiovascular risk, we found no significant relationship between plasma ADMA concentration and long-term cardiovascular outcome for the cohort at large. This apparent lack of association could be attributed at least in part to a substantial interaction of smoking status, plasma ADMA concentration, and cardiovascular risk. When accounting for this interaction, our data actually provide first evidence that ADMA is an independent risk marker in healthy nonsmoking men.

R.H.B. and R.M. have filed patents related to NOS inhibitors.

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