Relationship Between Obesity, Smoking, and the Endogenous Nitric Oxide Synthase Inhibitor, Asymmetric Dimethylarginine

Hilde M.A. Eid, Harald Arnesen, Elsa M. Hjerkinn, Torstein Lyberg, and Ingebjørg Seljeflot

We investigated the levels of asymmetric dimethylarginine (ADMA), an important endogenous inhibitor of nitric oxide (NO), as related to metabolic risk factors known to contribute to atherosclerotic disease. Dimethylarginines were analysed in a cross-sectional study of 563 elderly high-risk men (70 ± 6 years). ADMA and the L-arginine/ADMA (L-arg/ADMA) ratio were highly significantly correlated with several metabolic risk factors. However, only the association with body mass index (BMI) remained significant after adjustment for inter-related variables. When analyzing the results according to being overweight or not, ADMA levels were independently significantly higher ($P = .05$) and the L-arg/ADMA ratios were significantly lower ($P < .008$) in individuals with high BMI (≥26 kg/m^2, median value) as compared with subjects with low BMI. ADMA levels were furthermore significantly lower ($P = .037$) and L-arginine and the L-arg/ADMA ratios were significantly higher ($P = .004$ and $P = .001$, respectively) in smokers compared with nonsmokers, the latter being independent of other risk factors. The strong relationship found between BMI and plasma levels of ADMA and the L-arg/ADMA ratio indicate a link to endothelial dysfunction in overweight subjects. The beneficial dimethylarginine profile observed in smokers in this elderly population is not easily explainable and should be further investigated.

ENDOTHELIAL DYSFUNCTION is an early abnormality in the pathogenesis of atherosclerosis. Central to the development of endothelial dysfunction is reduced bioactive endothelial nitric oxide (NO). NO is synthesized from L-arginine by NO-synthase (NOS), and the endothelial derived NO, is beyond being an endogenous vasodilator, important in maintenance of cardiovascular homeostasis.[1-4] Alterations of the NOS pathway and impairment of NO-dependent vasodilation has been linked to several well-known atherogenic risk factors, including hypercholesterolemia, hypertension, diabetes, smoking, and ageing.[5]

Impaired vasodilation has been suggested to be caused by inhibition of NO generation by the amino acid, asymmetric dimethylarginine (ADMA), a recently described important endogenous competitive inhibitor of NOS.[6] ADMA has also been shown to increase oxidative stress by uncoupling of electron transport between NOS and L-arginine, and hence decrease both the production and availability of endothelium-derived NO.[7]

ADMA is constantly produced in the course of normal protein turnover in many tissues, including vascular endothelial cells, and is derived from hydrolysis of methylated proteins. It appears that both synthesis and degradation of ADMA and other methylarginines, ie, symmetric dimethylarginine (SDMA) and monomethylarginine, are highly actively regulated,[7] and dysregulation of either of these pathways may result in increased levels of free methylarginines.[8,9] Renal impairment has also been shown to reduce ADMA clearance resulting in ADMA accumulation.[6,10] Several studies furthermore support the view that the ratio between L-arginine and ADMA (L-arg/ADMA ratio) is important for the regulation of endothelial NOS activity.[11,12]

Elevated ADMA levels have been shown to antagonize the endothelium-dependent vasodilation in humans.[6,13,14] Increased ADMA levels have furthermore been reported to be implicated in the pathogenesis of conditions affecting the cardiovascular system, like diabetes mellitus,[15,16] and disorders related to the insulin resistance syndrome, ie, dyslipidemia, hyperglycemia, and essential hypertension.[9,13,15,17,18] Of note, Stuhlinger et al[19] recently observed a close relationship between ADMA levels and insulin resistance, independent of other risk factors. ADMA has furthermore been shown independently of other risk factors to be associated with intima media thickening in individuals with varying risk for atherosclerosis.[20]

The present cross-sectional study was undertaken to further investigate the influence of plasma ADMA levels on different metabolic disorders known to contribute to atherosclerotic disease states with the hypothesis that diabetes, insulin resistance, overweight, and smoking might be important determinants. A larger population of elderly men at high risk for coronary heart disease was investigated.

MATERIALS AND METHODS

Study Design and Study Population

This is a follow up study of participants from the Oslo Diet and Anti-smoking study performed 1972 to 1977,[21] comprising 1,232 men with hypercholesterolemia (total cholesterol > 6.45 mmol/L, 80% smokers). The survivors of this population were 25 years later invited to participate in the Diet and Omega-3 Intervention Trial on Atherosclerosis (DOIT), a 3-year intervention trial aimed at investigating the effects of omega-3 fatty acid supplementation and/or dietary intervention on markers of atherosclerosis.[22] Altogether, a total of 563 subjects, age 64 to 76 years, were included in the DOIT study. The present investigation is a cross-sectional study, based on results from the baseline examination. The study was approved by the Regional Ethics Committee, and all subjects gave their written informed consent.

From the Center for Clinical Research and the Department of Cardiology, Ullevaal University Hospital, Oslo, Norway.

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Address reprint requests to Hilde M.A. Eid, MSc, Department of Surgery, 4th floor, Ullevaal University Hospital, NO-0407, Oslo, Norway.

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Blood Sampling

Venous blood samples were collected in an overnight fasting state at 7:30 AM to 10 AM. EDTA plasma (0.34 mol/L EDTA-K3) was prepared for determination of the dimethylarginines and oxidized low-density lipoprotein cholesterol (oxLDL-C), whereas serum was used for nitrate/nitrite (NOx), thiobarbituric-acid-reacting substances (TBARS), insulin, glucose, and lipid analyses. Plasma and serum were kept frozen at −70°C for batch analysis of the dimethylarginines, oxLDL-C, NOx, and TBARS.

Laboratory Analyses

Total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), triglycerides (TG), glucose, and glycosylated hemoglobin (HbA_{1c}) were determined by conventional methods. LDL-C was calculated according to Friedewald's formula. TBARS was determined as previously described.[16]

Insulin was analyzed using a competitive radioimmunoassay (RIA) kit from Linco Research, St Charles, MO. This method is based on a double-bounded antibody (PEG) technique.

OxLDL-C was measured with an enzyme-linked immunosorbent assay (ELISA) kit from Mercodia AB, Uppsala, Sweden. The method is based on a direct sandwich technique in which 2 monoclonal antibodies are directed against separate antigenic determinants on the oxidized apolipoprotein B molecule.

NOx was analyzed using Total Nitric Oxide Assay kit (R&D System Europe, Abingdon, UK). Briefly, this assay is based on the enzymatic conversion of nitrate to nitrite by nitrate reductase. The reaction was followed by a colorimetric detection (540 nm) of nitrite as an azo dye product of the Griess Reaction. To minimize interference with plasma proteins, the samples were ultrafiltrated through a 12-kDa cut-off filter (VectaSpin Micro 12K MWCO; Whatman International, Maidstone, England) prior to the analysis of NOx.

Plasma concentration of L-arginine, ADMA, and SDMA were measured by high-performance liquid chromatography (HPLC) and precolumn derivatization with *o*-phthaldialdehyde (OPA) (Sigma Chemicals, St Louis, MO) as previously described in detail.[16]

Clinical Manifestations

Diabetes included individuals with manifest diabetes and/or fasting glucose > 7 mmol/L. Insulin resistance was estimated according to a homeostasis model assessment (HOMA) score, calculated with the following formula: (fasting insulin/7.2)/(22.5/fasting glucose), as described by Matthews et al.[23]

Subjects were defined as overweight if they had BMI ≥ 26 kg/m^2 (median value of the population). Smoking habits were recorded as current smokers or not.

Verified cardiovascular disease state (CVD, including individuals who had suffered an acute myocardial infarction) and treated hypertension were also recorded.

Statistical Analysis

As several variables were skewly distributed, data are presented as medians and 25, 75 percentiles throughout. For demographic variables, proportions are given. Correlations between variables were tested by Spearman correlation method. Mann Whitney rank sum test was used for comparison between groups. Multiple linear regression analyses were performed to evaluate the independent relationship between ADMA and L-arg/ADMA ratio and relevant metabolic risk factors (see Table 2). Serum lipids and age were not included in the model owing to the use of statins and the rather narrow range of age. We used a multiple logistic regression model to adjust for differences in risk factors when comparing the clinical manifestation groups. As insulin and the HOMA score were highly intercorrelated, only insulin was included in the regression models. Skewed variables were log-transformed before being entered in the regression models. The level of statistical significance was set at $P < .05$. The SPSS 11.0 (SPSS, Chicago, IL) software package was used for statistical analysis.

Table 1. Clinical Characteristics, Use of Medication, and Fasting Laboratory Variables in the Study Population (n = 563)

Age (yr) (range)	70 (64-76)
Verified CVD (%)	28
Diabetes (%)	15
Hypertension (%)	30
Smokers (%)	34
Medications (%)	
Statins	27
Acetylsalicylic acid	26
Beta-blockers	16
ACE inhibitors	13
Nitrates	9
Systolic blood pressure (mm Hg)	148 (135, 160)
Diastolic blood pressure (mm Hg)	83 (76, 91)
Body mass index (kg/height [m]^2)	26.5 (24.1, 28.7)
TC (mmol/L)	6.3 (5.7, 7.0)
HDL-C (mmol/L)	1.4 (1.1, 1.6)
LDL-C (mmol/L)	4.1 (3.5, 4.7)
Ratio TC/HDL-C	4.5 (3.7, 5.5)
TG (mmol/L)	1.5 (1.1, 2.0)
oxLDL-C (U/L)	67 (54, 82)
Glucose (mmol/L)	5.6 (5.3, 6.2)
HbA_{1c} (%)	5.6 (5.3, 5.9)
Insulin (pmol/L)	117 (93, 153)
HOMA score	4.1 (3.2, 5.6)
TBARS (μmol/L)	1.20 (0.96, 1.60)
Creatinine (μmol/L)	88 (80, 97)
ADMA (μmol/L)	1.39 (1.12, 1.76)
SDMA (μmol/L)	0.23 (0.17, 0.33)
L-arginine (μmol/L)	86 (76, 97)
Ratio L-arg/ADMA	62 (49, 77)
NOx (μmol/L)	26.1 (21.8, 34.5)

NOTE. Median values (25, 75 percentiles) or proportions are given.

RESULTS

Clinical characteristics, use of medication and laboratory variables for the total study population are presented in Table 1. As can be seen, 28% had CVD, 15% had diabetes, 34% were current smokers, 30% had treated hypertension, and 27% were statin users.

Correlations

The coefficients of correlations between ADMA levels, L-arg/ADMA ratio, and other relevant risk factors are shown in Table 2. Statin users were excluded from analyses including serum lipids. ADMA was significantly correlated with BMI ($P = .006$), insulin ($P < .001$), the HOMA score ($P < .001$), TC/HDL-C ratio ($P = .006$), and inversely with HDL-C ($P = .008$). Borderline significant correlation was observed also for TG ($P = .048$). Insulin and the HOMA score were significantly and inversely correlated with the L-arg/ADMA ratio (both $P < .001$). The ratio was furthermore negatively correlated with BMI ($P < .001$), TG ($P = .037$), TC/HDL-C ratio ($P = .011$), and TBARS ($P = .014$), whereas a positive correlation was

Table 2. Correlations Between ADMA and L-arg/ADMA Ratio and Relevant Risk Variables in the Total Population

	ADMA		Ratio L-arg/ADMA	
	r	*P*	*r*	*P*
BMI (kg/m^2)	0.12	.006†	−0.15	<.001‡
Glucose (mmol/L)	0.068	.11	−0.074	.081
HbA_{1c} (%)	0.061	.15	−0.064	.13
Insulin (pmol/L)	0.15	<.001	−0.15	<.001
HOMA	0.16	<.001	−0.16	<.001
TG (mmol/L)*	0.098	.048	−0.10	.037
HDL-C (mmol/L)*	−0.13	.008	0.13	.009
r TC/HDL-C*	0.14	.006	−0.13	.011
LDL-C*	0.024	.62	0.032	.53
oxLDL-C (U/L)*	−0.023	.64	0.022	.66
TBARS (μmol/L)	0.061	.15	−0.11	.014†
NOx (μmol/L)	−0.063	.14	0.019	.64
Creatinine (μmol/L)	0.083	.049	−0.029	.49
SBP (mm Hg)	0.050	.24	−0.026	.54
DBP (mm Hg)	0.055	.19	−0.057	.18

*Without statin administration.

†$.05 \le P < .10$; ‡$P < .01$, adjusted for related risk variables with log ADMA or log L-arg/ADMA ratio as dependent variables.

observed for HDL-C ($P = .009$). No correlations were found between blood pressure or age and ADMA or L-arg/ADMA ratio.

Multiple regression analyses were performed to define the independent relationship between ADMA levels, the L-arg/ADMA ratio, and various risk markers (Table 2). As shown, the L-arg/ADMA ratio was significantly and independently related to BMI ($P = .003$) and TBARS ($P = .050$), whereas the relationship to insulin was no longer present. ADMA was independently borderline significant related to BMI, but not to other metabolic risk factors.

Dimethylarginines and Clinical Manifestations

Median plasma concentration of ADMA in the total population (n = 563) was 1.39 μmol/L and the L-arg/ADMA ratio was 62 (Table 1). There were no differences in ADMA levels or the L-arg/ADMA ratio in subjects treated with statins, angiotensin-converting enzymes (ACE) inhibitors, β blockers, or nitrates when compared with untreated individuals. Individuals with diabetes had borderline significantly higher ADMA levels than the nondiabetics (1.47 *v* 1.38 μmol/L, $P = .067$), but no significant difference in the L-arg/ADMA ratio could be demonstrated. We observed no differences in plasma ADMA levels or L-arg/ADMA ratios in subjects with or without CVD (1.38 *v* 1.39 and 61 *v* 62, respectively) or in the group of treated hypertensives compared with normotensives (1.35 *v* 1.39, and 62 *v* 61, respectively).

When dichotomizing the levels of BMI, significantly higher ADMA levels and significantly lower L-arginine and L-arg/ADMA ratio were observed in the individuals with high BMI (≥26 kg/m^2) as compared with subjects with low BMI (<26 kg/m^2) ($P = .002$, $P = .014$, and $P < .001$, respectively) (Table 3). The BMI levels were divided according to the World Health Organization (WHO) definition[24] of being "normal" (<25 kg/m^2) (n = 188), overweight (25 to 29.9 kg/m^2) (n = 284), or obese (≥30 kg/m^2)(n = 82), and the results were confirmed by showing increasing levels of ADMA and decreasing levels of L-arg/ADMA ratio through the BMI groups (Fig 1). Overweight subjects also had significantly higher TBARS levels ($P = .005$), but no differences in oxLDL-C or NOx concentrations were observed between the groups. Overweight subjects had furthermore significantly higher levels of TG (1.7 *v* 1.3 mmol/L, $P < .001$) and elevated rTC/HDL-C (4.8 *v* 4.2, $P < .001$). Fasting glucose, insulin, HbA_{1c}, and the HOMA score were also higher in subjects with high BMI ($P < .0001$ for all) as compared with subjects with low BMI. The number of statin users was similar in the 2 groups. After adjustment for confounding variables, ADMA was still significantly elevated and the L-arg/ADMA ratio significantly lower in overweight individuals ($P = .050$ and $P = .008$, respectively) (Table 3).

Plasma concentration of ADMA was significantly lower and L-arginine and the L-arg/ADMA ratio were significantly higher in smokers as compared with nonsmokers ($P = .037$, $P = .004$, and $P = .001$, respectively) (Table 3). Significantly higher levels of NOx could also be demonstrated in smokers ($P = .008$), whereas no differences in TBARS or oxLDL-C concentration were observed between these groups. BMI, insulin, HOMA score, and creatinine were significantly lower in smokers as compared with nonsmokers ($P < .001$, $P = .002$, $P = .001$, and $P = .004$, respectively), whereas serum lipids and glucose levels did not differ. After adjustment for confounding variables, smokers had still significantly elevated levels of L-arginine and L-arg/ADMA ratio ($P = .007$ and $P = .008$, respectively) as compared with nonsmokers, whereas the differences in ADMA and NOx levels were no longer statistically significant (Table 3).

Table 3. Fasting Laboratory Variables According to Overweight and Smoking

	BMI < 26 (n = 279)	BMI ≥ 26 (n = 275)	*P*	*P*1	Smoke$^-$ (n = 364)	Smoke$^+$ (n = 189)	*P*	*P*2
BMI (kg/m^2)	24 (23, 25)	28 (27, 30)	<.001		27 (25, 29)	25 (23, 27)	<.001	<.001
ADMA (μmol/L)*	1.31 (1.07, 1.72)	1.44 (1.19, 1.78)	.002	.050	1.42 (1.16, 1.77)	1.32 (1.05, 1.72)	.037	.14
L-arginine (μmol/L)	87 (78, 98)	85 (76, 96)	.014	.12	85 (75, 96)	88 (80, 101	.004	.007
Ratio L-arg/ADMA*	66 (50, 81)	59 (48, 72)	<.001	.008	60 (48, 75)	66 (53, 81)	.001	.008
NOx (μmol/L)	26.0 (21.6, 34.7)	26.1 (21.7, 34.5)	.39	.41	25.2 (21.4, 34.0)	28.6 (22.4, 36.8)	.008	.06
TBARS (μmol/L)	1.2 (0.9, 1.5)	1.3 (1.0, 1.7)	.005	.61	1.2 (1.0, 1.6)	1.2 (1.0, 1.7)	.91	.51

NOTE. Median values (25, 75 percentiles) are given. *P* refers to differences between groups; *P*1, adjusted for rTC/HDL-C, insulin, log triglycerides, glucose, SBP, DBP, and smoking; *P*2, adjusted for insulin, SBP, DBP, BMI, and creatinine.

*Log transformed.

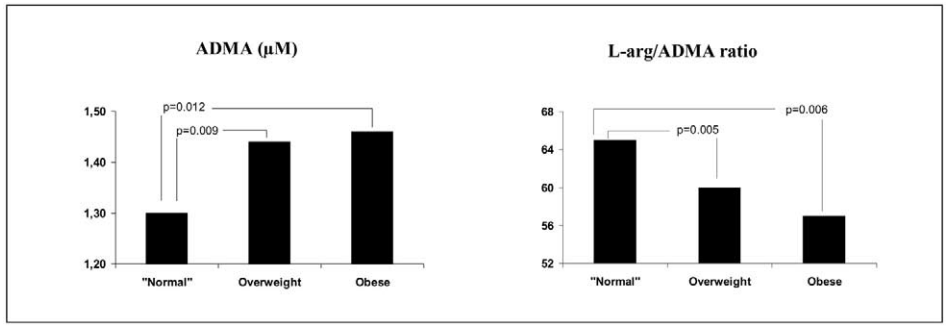

Fig 1. ADMA and L-arg/ADMA ratio according to the WHO criteria for being overweight or obese. Normal: BMI < 25 kg/m^2, overweight: 25 kg/m^2 ≤ BMI < 29.9 kg/m^2, obese: BMI ≥ 30 kg/m^2.

DISCUSSION

In the present study of 563 elderly men with high risk for coronary heart disease, we could show a close relationship between BMI and plasma levels of ADMA and the L-arg/ADMA ratio, independent of other metabolic risk factors. Overweight and obese subjects had significantly elevated ADMA levels and lower L-arg/ADMA ratio as compared with lean or normal individuals. Surprisingly, smokers, comprising 34% of the population, and with a smoking history of at least 25 years, had lower levels of ADMA and higher L-arg/ADMA ratio than nonsmokers.

Previous studies have shown concentrations of ADMA to be elevated in healthy old people compared with young and middle-aged subjects.[12] In the present study, ADMA levels in the total population were similar to what have been shown in subjects with hypercholesterolemia, with almost 2-fold higher levels than in young healthy subjects.[16] These levels of ADMA may have pathophysiologic significance, as they are within the range shown to inhibit the activity of NOS.[25]

Elevated ADMA levels have been shown in a wide range of disease states.[6,16,26,27] We could, however, not demonstrate any differences in ADMA levels or L-arg/ADMA ratio in subjects with or without CVD, treated hypertensives versus normotensives or diabetics versus nondiabetics in the present population. We could also not find any association between ADMA and systemic blood pressure, which has been described in some studies.[17,19,20] Our observations may be explained by the heterogeneity of this older population, survivors from a population at high risk for CVD, using a broad spectre of medication that may affect the levels of ADMA and L-arginine through mechanisms not known, although no influence of the recorded medications could be seen. The L-arginine concentrations observed in the present population were close to values obtained in healthy, younger subjects.[16]

There is growing evidence that endothelial dysfunction is central in the insulin resistance syndrome and type 2 diabetes,[28,29] in which it has been shown that the release and/or bioavailability of NO are diminished.[30,31] Several cardiovascular risk factors are associated with reduced sensitivity to insulin, and recently elevated ADMA concentrations were linked to the metabolic syndrome. Stühlinger et al[19] observed a strong relationship between insulin resistance and plasma concentrations of ADMA. The present results from our larger population are in accordance with these findings, showing highly significant correlations between ADMA and insulin and the HOMA score, an alternative to more sophisticated techniques for in vivo evaluation of insulin sensitivity.[23] Furthermore, these variables were inversely correlated with the L-arg/ADMA ratio. These associations were, however, not independent of the multiple risk components in this syndrome. We did, nevertheless, observe highly significant associations between ADMA and L-arg/ADMA ratio and BMI, another well-known component of the insulin resistance syndrome, especially the L-arg/ADMA ratio, remaining significant also after adjustment for related risk factors. This close association was confirmed when we divided the individuals according to being overweight or not, revealing increased ADMA levels and especially reduced L-arg/ADMA ratio in overweight subjects. Although some reports on the relationship between BMI and the activity of NO-dependent pathways are contradictory,[32,34] NO-dependent vasodilation has been shown to be impaired in obese insulin-resistant patients.[35] According to the present results, this may be discussed as a result of elevated ADMA levels in such patients. Overall, it seems that the imbalance between L-arginine and ADMA levels should be included in the cluster of risk factors in the insulin resistance syndrome, although direct mechanisms are still unknown. A possible direct link between obesity and ADMA might be through increased inflammatory activity in adipocytes, especially with increased synthesis of tumor necrosis factor-α (TNF-$\alpha\alpha$),[36,37] which, in turn, has been shown to increase the expression of ADMA in endothelial cell cultures.[38] We could furthermore show that the degree of peroxidation, unspecifically assessed as TBARS, was elevated in overweight individuals. TBARS was also found to be independently inversely related to the L-arg/ADMA ratio. In this context, it is interesting that the ADMA catabolic enzyme protein arginine N-methyltransferase I (PRMT I) and the metabolic enzyme, dimethylarginine dimethylaminohydrolase (DDAH) have both been shown to be redox-sensitive.[8] Recent in vitro studies have demonstrated that oxidative stress is associated with increased concentrations of ADMA in human endothelial cell supernatants.[8] Thus, the linkage between obesity and endothelial dysfunction might be drawn through inflammation, oxidation, and increased inhibition of NO synthesis, the latter by an imbalance between ADMA and L-arginine. In line with this, an association between ADMA or L-arg/ADMA ratio and the levels of LDL-C and oxLDL-C might be expected, as an oxLDL-C regulating pathway of ADMA has been focused.[8,9,39] There are, however, conflicting reports regarding the relationship between ADMA and LDL-C.[13,16,40] We could not demonstrate any correlations

to the levels of either oxLDL-C or LDL-C, whereas strong associations were observed between both ADMA and the L-arg/ADMA ratio and HDL-C. Owing to the use of statins in the present population, independent relationships are not easy to rule out.

Although there is a strong association between cigarette smoking and the development and progression of atherosclerosis, there are limited data about the direct influence of tobacco consumption. The pathophysiologic mechanisms underlying these associations are therefore still under debate. Smoking is discussed to cause endothelial dysfunction by increased oxidative damage brought about by radical oxygen species. There are limited data regarding the influence of smoking on ADMA levels.[41] In the present larger population, we surprisingly observed lower ADMA levels, higher L-arginine, and L-arg/ADMA ratio in smokers versus nonsmokers, independent of confounding variables including BMI. We find these observations interesting, and it seems likely that the relatively high levels of L-arginine may be important. L-arginine supplementation has been shown to limit both native LDL-C and oxLDL-C–induced endothelial dysfunction.[42] The present results were to some degree supported by the finding of higher levels of the NO metabolites, Nox, in smokers when compared with nonsmokers. This measurement of NOx by the Griess reaction reflects both NO metabolites, as well as bioactive NO, thus bioactive NO cannot be exactly estimated. Our results are not in line with some previous reports regarding smoking and NOx in other populations. It was shown that acute cigarette smoking in young healthy smokers gave a temporary decrease in nitrite and nitrate,[43] and in the study of Mazzone et al,[44] they showed reduced levels of NOx in their population of hypertensive smokers. It should be noted that the smokers in the present population had been chronic smokers for at least 25 years. It could furthermore be discussed that our population might have some protection in scavenging free radicals, thus not being exposed to oxidative stress as supposed, as the levels of TBARS and oxLDL-C did not differ between smokers and nonsmokers. Moreover, it should be emphasized that the present population is survivors from a high-risk population and might thus be considered to have some protective traits. One might again speculate on the potential beneficial effect of higher levels of L-arginine.

In conclusion, the strong relationship between BMI and plasma levels of ADMA and the L-arg/ADMA ratio shown indicate a close link to endothelial dysfunction in overweight subjects. ADMA and L-arg/ADMA ratio should therefore be included in the cluster of metabolic risk factors known to contribute to atherosclerotic disease. The beneficial dimethylarginine profile in smokers is not easily explainable and should be further investigated.

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