

Plasma concentration of asymmetric dimethylarginine (ADMA) predicts cardiovascular morbidity and mortality in type 1 diabetic patients with diabetic nephropathy

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ABSTRACT

Objective: To investigate whether circulating ADMA levels are predictive of cardiovascular events, decline in glomerular filtration rate (dGFR), end-stage renal disease (ESRD), and all-cause mortality in type 1 patients.

Research Design and Methods: A prospective observational follow-up study; including 397 type 1 patients with overt diabetic nephropathy (243 men; age 42.1 ± 10.5 years (mean \pm SD); GFR 76 ± 34 ml/min/1.73 m²) and a control group of 175 patients with longstanding type 1 and persistent normoalbuminuria (104 men; age 42.7 ± 9.7 years; duration of diabetes 27.7 ± 8.3 years). Patients were followed for 11.3 (0.0-12.9) years (median (range)) with yearly measurements of GFR (⁵¹Cr-EDTA plasma clearance) in patients with diabetic nephropathy. Endpoints were fatal and non-fatal CVD, dGFR, ESRD, and all-cause mortality.

Results: Among patients with diabetic nephropathy, 37 (19.4 %) patients with ADMA levels below the median compared to 79 (43.4 %) patients above the median suffered a major cardiovascular event during the follow-up period ($p < 0.001$). This effect persisted after adjustment for conventional CVD risk factors including baseline GFR (adjusted hazard ratio (HR) for elevated ADMA of 2.05 (1.31; 3.20), $p = 0.002$). Furthermore, elevated ADMA levels predicted an increased rate of decline in GFR, development of ESRD, and all-cause mortality ($p < 0.001$). After adjustment for well known progression promoters including baseline GFR HR (adjusted) was 1.85 (0.99; 3.46); $p = 0.055$ for ESRD comparing upper and lower median ADMA levels.

Conclusions: Plasma ADMA levels predict fatal and non-fatal cardiovascular events in patients with type 1 nephropathy. Furthermore, increased ADMA levels tended to contribute to increased risk of progressive diabetic kidney disease.

Diabetes mellitus is associated with accelerated atherosclerosis and an increased risk of clinical cardiovascular disease (CVD), which has become the major cause of morbidity and mortality among patients with diabetic nephropathy(1). Endothelial dysfunction is characterized by impaired nitric oxide (NO)-mediated vascular response and is associated with development of atherosclerosis and other diabetic vascular complications in diabetes(2).

Asymmetric dimethylarginine (ADMA) is a naturally occurring endogenous L-arginine metabolite with the capacity to inhibit all three isoforms of nitric oxide synthases (NOS). ADMA, and to a greater extent its stereoisomer symmetrical dimethylarginine (SDMA), which has no effect on NO-synthases, have been reported to accumulate in patients with renal failure(3). Although ADMA is excreted by the kidneys to some extent, the major metabolic pathway is degradation by the dimethylarginine dimethylaminohydrolases (DDAH1 and DDAH2) into dimethylamine and L-citrulline(4,5). In vitro, hyperglycaemia impairs DDAH activity in vascular smooth muscle cells and the endothelium thereby contributing to elevated ADMA levels among diabetic patients(6).

Chronic elevation of ADMA in animals causes atherosclerotic lesions and renal damage as a consequence of reduced NO generation(7). This suggests an important role of ADMA in explaining the relationship between endothelial dysfunction, atherosclerosis, and diabetic nephropathy.

ADMA has been shown to be increased in conditions such as impaired renal function(3,8), diabetes mellitus(9), hypertension(10) and diabetic nephropathy(11). More recently, ADMA levels have been shown to be predictive of CVD and all-cause

mortality in predominantly non-diabetic chronic kidney disease(12), end-stage renal disease (ESRD)(8), and coronary artery disease (CAD)(13-15).

Therefore, we investigated the predictive value of circulating ADMA levels on risk of fatal and non-fatal CVD, progression to ESRD, rate of decline in GFR, as well as all-cause mortality in a well-characterized population of type 1 diabetic patients with or without diabetic nephropathy.

RESEARCH DESIGN AND METHODS

Patients. During 1993, 198 type 1 diabetic patients with diabetic nephropathy, whose glomerular filtration rate had been measured during the same year, were recruited from the outpatient clinic at Steno Diabetes Center for a case-control study. Simultaneously, a group of 192 patients with long lasting type 1 diabetes (≥ 15 years, mean (SD) 27.7 ± 8.3 years) and persistent normoalbuminuria were recruited as controls.

Since then, all type 1 diabetic patients newly referred for measurement of kidney function have been invited to participate in an ongoing study of the genetics of diabetic nephropathy, and thus the original cohort of type 1 diabetic patients with diabetic nephropathy was expanded to 408 patients. Diabetic nephropathy is diagnosed clinically as persistent macroalbuminuria (>300 mg/24h) in at least two out of three consecutive 24 hours urine collections, in the presence of diabetic retinopathy and the absence of other kidney or urinary tract disease(16). In case no signs of retinopathy were observed a kidney biopsy was required for diagnosis of diabetic nephropathy. Patients with urinary albumin excretion rate in the microalbuminuric range were not included. In 2002 the treatment recommendations at SDC were extended to include statins and low-

dose aspirin for all patients with diabetic nephropathy.

Plasma ADMA levels was determined and follow-up data was available for 397 (97 %) of patients with diabetic nephropathy and 175 (91 %) of patients with type 1 diabetes for more than 15 years and persistent normoalbuminuria (<30 mg/24h).

Measurements of ADMA. Plasma concentrations of ADMA, SDMA, and L-arginine were determined simultaneously by high-performance liquid chromatography as described previously(17). In brief, sample cleanup was performed by solid-phase extraction on polymeric cation-exchange extraction columns using monomethylarginine as internal standard. After derivatization with ortho-phthaldialdehyde reagent containing 3-mercaptoproprionic acid, analytes were separated by isocratic reversed-phase high-performance liquid chromatography with fluorescence detection. Analytical recovery was 98-102 % and the interassay coefficient of variation was <3 %. The plasma concentrations of ADMA and SDMA were measured in 2002, and do not change upon prolonged storage(18). In a recent review a reference between 0.4 and 0.6 μ mol/l was suggested(19) – in our hands we measured a mean ADMA concentration in the general population (n=2311) of 0.5 (range:0.39-0.63) μ mol/l((18).

Follow-up. In 1993, a prospective observational follow-up study in case-control design with a specified endpoint of cardiovascular morbidity and mortality was initiated at Steno Diabetes Center. From this cohort, all patients were followed until death, to the last visit at Steno Diabetes Center, or until the 1st of September 2006 by tracing through patient records during autumn 2006. The primary endpoint was time to major fatal- and non-fatal cardiovascular

event; secondary endpoints were dGFR, time to ESRD, and all-cause mortality.

Information about date of non-fatal CVD events and date of ESRD defined as kidney transplantation or dialysis were obtained from patient records or discharge letters from other hospitals. Major cardiovascular events were diagnosed as stroke, myocardial infarction, Coronary artery bypass graft (CABG) and/or percutaneous coronary intervention (PCI).

Glomerular filtration rate (GFR) was measured annually in patients with diabetic nephropathy after a single injection of 3.7 MBq ⁵¹Cr-EDTA by determination of radioactivity in venous blood samples taken 180, 200, 220 and 240 minutes after injection(20). The results were standardized for 1.73 m² body surface area, using the patient surface area at the initial GFR measurement. The mean individual day-to-day variation in GFR is 4 % in our laboratory. Linear regression analysis of all GFR determinations in each individual was used to estimate the rate of decline in kidney function. In the normoalbuminuric patients, GFR was estimated by the MDRD formula(21).

All patients' vital status was traced through the national register at the end of 2006. If a patient had died before the 1st of September 2006 the date of death was recorded and information on cause of death was obtained from the death certificate. Two observers reviewed all death certificates independently and the primary cause of death was recorded. Additional available information from necropsy reports was included. All deaths were classified as cardiovascular deaths unless an unequivocal non-cardiovascular cause was established(22).

The study was performed in accordance with the Helsinki Declaration, the local ethics committee approved the study and all patients gave their informed consent.

Statistical Analysis. Normally distributed variables are given as means \pm SD, whereas non-normally distributed variables were log transformed before analysis and are given as medians (range). Comparisons between groups were performed using unpaired Student's t-test or ANOVA. A χ^2 -test was used to compare non-continuous variables.

All time-to-endpoint variables were analyzed using a log-rank test and displayed on Kaplan-Meier plots. The relations between ADMA and endpoints during follow-up were investigated in either a Cox regression enter model (fatal and non-fatal CVD, ESRD, and overall mortality) displayed as unadjusted and adjusted hazard ratios given with 95 % confidence interval or a multiple linear regression analysis (dGFR). A two-tailed p-value of 0.05 or less was considered statistically significant.

All calculations were performed using a commercially available program (SPSS for Windows, version 13.0, Chicago, IL, USA).

RESULTS

The baseline clinical and biochemical characteristics of study groups are shown in Table 1. Plasma ADMA levels was determined and follow-up data available in 397 (97%) of patients with diabetic nephropathy and 175 (91%) of patients with type 1 diabetes for more than 15 years and persistent normoalbuminuria (<30 mg/24h). In patients with diabetic nephropathy, mean plasma ADMA concentration was higher than in the normoalbuminuric group (0.46 ± 0.08 vs. 0.40 ± 0.08 $\mu\text{mol/l}$, respectively, $p < 0.001$). In parallel to ADMA, plasma SDMA concentrations were elevated in diabetic patients with nephropathy as compared with normoalbuminuric patients (0.59 (0.28 - 4.04) vs 0.41 (0.28 - 0.84) $\mu\text{mol/l}$; $p < 0.001$)(11).

The patients were followed prospectively for a median follow-up period until death or last visit of 11.3 (0.0-12.9) years. Among the group of patients with diabetic nephropathy, 37 (19.4 %) patients with ADMA levels below the median compared to 79 (43.4 %) patients above the median suffered a fatal or non-fatal major cardiovascular event during the follow-up period ($p < 0.001$) as shown in figure 1. Cox regression analysis revealed an unadjusted HR for fatal or non-fatal major cardiovascular event of 2.58 (1.75; 3.81), A model including the baseline variables sex, age, HbA_{1c}, systolic blood pressure, GFR, cholesterol, smoking status, previous CVD events and anti-hypertensive treatment (AHT) as covariates, revealed an adjusted HR of 2.05 (1.31; 3.20), ($p = 0.002$). Plasma NT-proBNP was positively correlated with ADMA and therefore further adjustment for NT-proBNP was performed. The adjusted HR was only slightly changed: 2.33 (1.30; 4.20), ($p = 0.005$). Entering hsCRP into the model had no impact on HR.

In analyses of fatal and non-fatal cardiovascular events separately, plasma ADMA predicted non-fatal major cardiovascular events with an adjusted HR of 2.28 (1.31 – 3.96), $p = 0.004$ and only borderline cardiovascular mortality: HR (adjusted): 1.78 (0.97 – 3.27), $p = 0.07$.

The mean (SD) rate of decline in GFR was 3.7 (3.7) ml/min/1.73 m²/year for patients with ADMA levels below the median compared to 4.9 (4.3) ml/min/1.73 m²/year for patients with ADMA levels above the median ($p < 0.001$) as shown in figure 2. This corresponds to 1.2 (0.4; 2.0) ml/min/1.73 m²/year, ($p = 0.004$) increased rate of decline in GFR in patients with ADMA levels above the median. When including the baseline variables sex, age, HbA_{1c}, systolic blood

pressure, GFR, cholesterol, and AHT as covariates in the analysis elevated ADMA levels still predicted a faster rate of decline in GFR during follow-up (0.9 (0.0; 1.75); ($p= 0.051$)). Furthermore, the risk of developing ESRD during the follow-up period was examined and 19 (10 %) patients with ADMA levels below the median reached ESRD during follow-up versus 51 (26 %) patients above the median ($p < 0.001$) corresponding to an unadjusted HR for risk of developing ESRD of 3.20 (1.89; 5.43). After correction for the above mentioned progression promoters the effect of the ADMA levels were attenuated (adjusted HR of 1.85 (0.99; 3.46), ($p= 0.055$)).

In total, 126 (33 %) of the patients with diabetic nephropathy died during follow-up, half of them due to cardiovascular causes. Of these, 50 (25 %) patients with ADMA levels below the median died during follow-up versus 76 (39 %) patients above the median ($p= 0.004$) with an unadjusted HR of overall mortality of 1.67 (1.17; 2.39). The difference in mortality reflects an increased mortality due to cardiovascular causes of 10 % and 24 % below and above the median level of ADMA, respectively. When including the baseline variables sex, age, HbA_{1c}, systolic blood pressure, GFR, smoking, cholesterol, previous CVD, and AHT as covariates, plasma ADMA was no longer predictive of all-cause mortality ($p= 0.70$).

In univariate analyses, plasma levels of SDMA at baseline predicted fatal or non-fatal CVD, all-cause mortality, decline in GFR and development of ESRD among the group of patients with diabetic nephropathy ($p < 0.01$). However, in multivariate analyses these associations were no longer statistically significant. Accordingly, the level of L-arginine predicted cardiovascular outcomes in univariate analysis ($p < 0.05$), but these findings

disappeared after adjustment for other cardiovascular risk factors. The ADMA/L-arginine ratio did not predict outcome, NS.

Among the 175 patients with persisting normoalbuminuria, a relatively low number of events defined as either fatal or non-fatal major cardiovascular events ($n= 21$) or all-cause mortality ($n= 20$) occurred. In normoalbuminuric patients with ADMA levels below the median, 12 (14 %) patients compared to 9 (11 %) patients above the median suffered a fatal or non-fatal major cardiovascular event ($p= 0.56$). During the follow-up period, 6 (7 %) patients with ADMA levels below the median compared to 14 (16 %) patients above the median died during the follow-up period ($p= 0.050$) revealing an unadjusted hazard ratio of 2.52 (0.97; 6.56).

CONCLUSIONS

In the present prospective observational study including 397 type 1 diabetic patients with diabetic nephropathy plasma ADMA levels predicted fatal and non-fatal CVD in patients with type 1 diabetes and diabetic nephropathy. When controlling for conventional CVD risk factors as well as baseline GFR, HbA_{1c} and use of antihypertensive treatment in multivariable models ADMA retained power to predict CVD morbidity and mortality in our study. Furthermore, elevated levels of plasma ADMA were related to increased loss of renal function (dGFR) and development of ESRD. In the multivariate analyses the association of plasma ADMA and dGFR as well as ESRD tended to be statistically significant.

Our study, which includes a reasonable sized ($n= 572$), clinically well-characterized case-control study population of type 1 diabetic patients with and without diabetic nephropathy and a median follow-up period of more

than 11 years, confirms and extends the finding of several previous studies of ADMA in relation to CVD risk. Previously, in a relatively small cohort (n= 125) of micro- and macroalbuminuric patients with type 2 diabetes, elevated ADMA levels were associated with increased cardiovascular risk and predicted future cardiovascular events(23). Similarly, among 225 primarily non-diabetic haemodialysis patients, plasma ADMA was found to be a strong and independent predictor of incidence of major cardiovascular events and death of any cause(8). As in previous studies, our study was designed to do baseline prediction based on a single measurement of ADMA and thus we can not exclude a regression dilution bias. Future studies including multiple measurements of ADMA during follow-up will provide useful information on disease progression in diabetic as well as non-diabetic populations.

Among several confounding factors, GFR may be particularly important since impaired renal function represents a cardiovascular risk factor by itself and hence high ADMA may merely indicate reduced renal excretion of ADMA due to renal dysfunction. However, in our study we adjusted for conventional CVD covariates including GFR at baseline. Furthermore, plasma levels of the stereoisomer symmetrical dimethylarginine (SDMA) previously reported to accumulate in patients with renal failure(3) was not associated with increased risk of cardiovascular morbidity and mortality, suggesting mechanisms other than renal dysfunction to account for the association between CVD risk and ADMA levels. ADMA is an endogenous competitive inhibitor of the enzyme NO synthase, a core element in the regulation of maintenance of vascular tone and structure of the endothelium. Chronic elevation of ADMA causes

atherosclerotic lesions in animals(7), implying a potential role of ADMA in the development of atherosclerosis in diabetic patients with proteinuria. Furthermore, Kielstein et al. showed that systemic administration of ADMA reduces NO generation, renal perfusion and sodium excretion in healthy humans. By causing significant renal vasoconstriction indicative of glomerular hypertension without affecting the renin-angiotensin system and sympathetic activity this points towards a direct effect of elevated ADMA levels on renal function(24). Alternatively, the raised ADMA levels predicting increased risk of CVD may simply be a marker of other important biologic process involved in the pathophysiology of endothelial dysfunction and renal damage. Although neither left ventricular mass or carotid intima-media thickness was measured in the present cohort, data from the literature indicates positive association between circulating ADMA levels and both these indicators of cardiovascular risk(25-28). Finally, the possibility remains that elevated ADMA may lead to accelerated cell senescence as previously suggested(29-31).

In our cohort of 397 type 1 diabetic patients with diabetic nephropathy the overall mortality among patients with baseline ADMA levels below the median was 25 % compared to 39 % for patients above the median reflecting an increased mortality due to cardiovascular causes. Hence, no difference in non-CVD related mortality was seen among the patients with baseline ADMA levels below and above the median level of ADMA. When including the baseline variables sex, age, HbA_{1c}, systolic blood pressure, GFR, smoking, previous CVD and AHT as covariates, plasma ADMA was no longer predictive of overall mortality.

Previously, we have in this population shown that circulating ADMA levels are elevated in patients with

diabetic nephropathy, even when the GFR is still within the normal range, compared to patients with persistent normoalbuminuria(11). Then, in a prospective observational design these patients were followed for more than 11 years with annual measurements of the plasma clearance of ⁵¹Cr-EDTA, an accurate and precise measure of GFR. The present data indicates that not only is ADMA elevated early in the development of diabetic nephropathy among type 1 diabetic patients, but in addition ADMA is predictive of a faster progression of diabetic nephropathy towards ESRD.

ADMA is synthesized by posttranslational methylation of arginine residues of proteins by the PRMT1 enzymes. In endothelial cells, PRMT expression levels have been shown to correlate with changes in ADMA release(32). Thus, the rate of ADMA generation could, at least partly, be regulated through alteration in PRMT1 expression caused by genetic factors. The major catabolic route of ADMA is degradation by the dimethylarginine dimethylaminohydrolases (DDAH1 and DDAH2) into dimethylamine and L-citrulline(4,5). Both isoforms of DDAH are highly expressed in the kidney(33) and hyperglycaemia has been shown to suppress DDAH activity in vitro leading to increased ADMA levels(6) possibly contributing to explain the association of ADMA levels and diabetic nephropathy. Recently, Jones et al. identified several functional genetic variants of the DDAH2 gene to be involved in DDAH expression(34). Leiper et al. report that Ddah1^{+/-} knock-out mice had reduced

expression of Ddah1 mRNA and DDAH1 protein, resulting in halving of DDAH activity and correspondingly increased plasma and tissue levels of ADMA compared with wild-type mice(35). The observation of familial clustering of diabetic nephropathy is suggestive of a genetic component involved in the development of this disease(36). Therefore, human association studies of genetic variants covering the PRMT and DDAH genes, ADMA levels, and diabetic heart and kidney disease are warranted.

At the moment, no specific ADMA lowering therapies are available. Previous studies have not found any effect on circulating ADMA after aggressive LDL lowering treatment with statins, whereas ACE inhibitors and angiotensin receptors blockers seem to lower ADMA in small studies of short duration, and thus needs to be confirmed(37).

In conclusion, plasma ADMA levels predict fatal and non-fatal cardiovascular events in patients with type 1 diabetic nephropathy. In addition, elevated ADMA levels suggest an increased risk of progressive diabetic kidney disease.

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TABLE 1. Baseline clinical and biochemical characteristics of the 572 patients with type 1 diabetes with and without diabetic nephropathy

	(n= 397)	(n=175)	
Sex (men/women)	243/154	104/71	0.38
Age (years)	42.1 ± 10.5	42.7 ± 9.7	0.55
Duration of diabetes (years)	28.3 ± 8.9	27.7 ± 8.3	0.41
BMI (kg/m ²)	24.3 ± 3.4	23.7 ± 2.5	0.03
HbA _{1c} (%)	9.4 ± 1.5	8.5 ± 1.1	<0.001
Urinary albumin excretion rate (mg/24 h)*	609 (10-14545)	8 (1-30)	-
Systolic blood pressure (mmHg)	145 ± 22	132 ± 18	<0.001
Diastolic blood pressure (mmHg)	82 ± 13	76 ± 10	<0.001
GFR (ml/min/1.73m ²)	76 ± 34	93 ± 15	<0.001
Serum creatinine (µmol/l)	102 (52-684)	76 (53-116)	<0.001
Serum cholesterol (mmol/l)	5.6 ± 1.2	4.8 ± 1.0	<0.001
Retinopathy (nil/simplex/proliferative)	6/129/262	61/95/19	<0.001
Previous CVD [†]	10.5 %	1.7 %	<0.001
Antihypertensive therapy (AHT)**	75 %	10 %	<0.001
ADMA (µmol/l)	0.46 ± 0.08	0.40 ± 0.06	<0.001
SDMA (µmol/l)	0.59 (0.28-4.04)	0.41 (0.28-0.84)	<0.001

Data are n, mean ± SD, or median (range). *Some patients with previously persistent macroalbuminuria at the time of investigation had a urinary albumin excretion rate below 300mg/24 h due to ongoing antihypertensive therapy. [†]Presence of previous CVD is defined as either stroke or myocardial infarction. ** In 2002 the recommendations at SDC were extended to include statins and low-dose aspirin for all patients with diabetic nephropathy.

FIGURE LEGENDS

Fig. 1. Kaplan-Meier curves of fatal and non-fatal cardiovascular events in 397 patients with type 1 diabetes and diabetic nephropathy according to the median baseline ADMA levels (0.46 $\mu\text{mol/l}$); ($p < 0.001$). The light gray line; ADMA levels above the median and the dark gray line; ADMA levels below the median. Hazard Ratio = 2.58 (1.75; 3.81), HR (adj.)= 2.05 (1.31; 3.20).

Fig. 2. Annual rate of decline in GFR (ml /min// 1.73 m²) in 397 patients with type 1 diabetes and diabetic nephropathy below and above the median (0.46 $\mu\text{mol/l}$) baseline ADMA levels, respectively ($p < 0.001$).

Figure 1

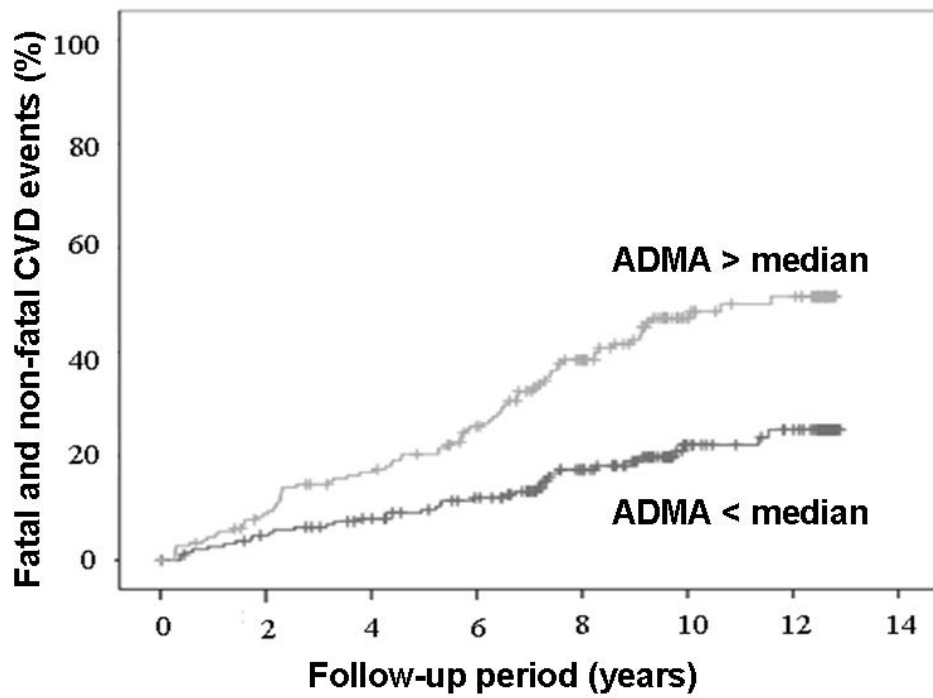


Figure 2

