Asymmetric Dimethylarginine and Progression of Chronic Kidney Disease: The Mild to Moderate Kidney Disease Study

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Reduced bioavailability of nitric oxide (NO) is thought to play an important role in progression of renal damage. The hypothesis that the endogenous NO synthase inhibitor asymmetric dimethylarginine (ADMA) is involved in progression of kidney disease was tested. Plasma ADMA concentrations and other putative progression factors were assessed in 227 relatively young patients (45.7 ± 12.6 yr) with nondiabetic kidney diseases and mild to moderate renal failure. Progression assessed as doubling of serum creatinine and/or renal replacement therapy was evaluated prospectively. Baseline plasma ADMA concentrations in renal patients correlated significantly with serum creatinine ($r = 0.595$), GFR ($r = -0.591$), age ($r = 0.281$), and proteinuria ($r = 0.184$; all $P < 0.01$). Patients who reached an end point during follow-up were significantly older ($P < 0.05$) and had significantly higher creatinine, ADMA, and parathyroid hormone blood concentrations and protein excretion rates at baseline, whereas GFR and hemoglobin were significantly lower (all $P < 0.01$). Cox regression analysis revealed baseline serum creatinine (odds ratio 2.00; 95% confidence interval [CI] 1.61 to 2.49; $P < 0.001$) and ADMA (odds ratio 1.47; 95% CI 1.12 to 1.93 for an increment of 0.1 μmol/L; $P < 0.006$) as independent predictors of disease progression. In patients with ADMA levels above median, progression was significantly faster ($P < 0.0001$), and their mean follow-up time to a progression end point was 52.8 mo (95% CI 46.9 to 58.8) as compared with 71.6 mo (95% CI 66.2 to 76.9) in patients with ADMA levels below the median. The endogenous NO synthase inhibitor ADMA is significantly associated with progression of nondiabetic kidney diseases. Lowering plasma ADMA concentrations may be a novel therapeutic target to prevent progressive renal impairment.

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Numerous experimental studies have revealed an important role for nitric oxide (NO) in progressive kidney damage (1–5). Apart from increased systemic BP, endothelial cell injury and dysfunction as a result of decreased local NO production may contribute to progression (1,4–8). Indeed, Kang *et al.* (4) administered an inhibitor of the NO synthase (NOS) to laboratory animals and observed significantly accelerated progression in addition to impairment of the angiogenic response and loss of the capillary endothelium, which was greater than expected for the increase in systemic BP. This finding and similar observations of other authors suggest an overriding role of local NO production in maintaining renal vascular endothelium (4–8).

Recently, endogenous NOS inhibitors such as asymmetric dimethylarginine (ADMA) have gained much attention. The role of increased plasma ADMA concentrations in endothelial dysfunction and vascular injury has been studied in various conditions such as pre-eclampsia, diabetes, stroke, and peripheral vascular and coronary heart disease (9–11). It has also been proposed that increased ADMA blood levels contribute to progression of chronic kidney disease, but so far only experimental data exist in support for this hypothesis (12). This notion is of considerable interest, because plasma ADMA concentrations were found to be already increased in early stages of renal disease, and the kidney itself seems to be an important organ of ADMA metabolism (13–15). Because in humans the role of ADMA in progression has not been explored so far, we assessed ADMA and other putative progression factors in 227 patients with nondiabetic kidney diseases and different degrees of renal dysfunction. Patients were thereafter followed prospectively for up to 7 yr. The primary study end point was doubling of serum creatinine and/or initiation of renal replacement therapy.

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Materials and Methods

Patients and Protocol

White male or female patients ($n = 227$) who were aged between 18 and 65 yr and had nondiabetic chronic kidney disease were recruited from eight nephrology departments in Germany, Austria, and South Tyrol as described earlier (16,17). The study was approved by the institutional ethics committees, and all patients gave written informed consent. Renal function had been stable for at least 3 mo before the baseline examination. Exclusion criteria were immunosuppressive agents, fish oil or erythropoietin, serum creatinine >6 mg/dl, diabetes, malignancy, liver or infectious disease, nephrotic syndrome (defined as daily proteinuria >3.5 g/1.73 m^2), organ transplantation, allergy to ionic contrast media, and pregnancy. The cause of kidney disease was glomerulonephritis in 97 patients (biopsy confirmed in 90), adult polycystic kidney disease in 37 patients, chronic interstitial nephritis in 24 patients, other types of kidney disease in 43 patients, and unknown in 26 patients.

For avoiding interobserver differences, all patients were recruited by one physician (Erich Kuen, Innsbruck), who visited all participating centers. Patient history, including smoking habits, was obtained by interview and confirmed by checking patient records. This was complemented by clinical examination that included assessment of body mass index (BMI) and BP. Hypertension was defined as BP >140/90 mmHg and/or antihypertensive medication. Antihypertensive drugs were taken by 179 (79%) patients: diuretics ($n = 83$; 37%), angiotensin-converting enzyme (ACE) inhibitors ($n = 123$; 54%), calcium channel blockers ($n = 78$; 34%), β receptor blockers ($n = 67$; 30%), and α-1 receptor blockers ($n = 36$; 16%). Blood samples for measurement of routine chemistry, insulin, intact parathyroid hormone (PTH), high-sensitivity C-reactive protein, and ADMA levels were taken after an overnight fast of at least 12 h. The samples were centrifuged immediately at 1500 × *g* and 4°C for 10 min. The supernatants were stored in aliquots at −80°C until further use. GFR was assessed in all patients using the iodothalamate clearance technique as described in detail elsewhere (16–18). Antihypertensive medication (if present) was withheld on the day of the study to minimize interference with measurements. Thereafter, patients were followed prospectively until the patient had doubling of serum creatinine, terminal renal failure necessitating renal replacement therapy, or reached the end of the 82-mo observation period.

Measurements and Calculations

Plasma concentrations of ADMA and its biologically inactive stereoisomer symmetric dimethylarginine (SDMA) were measured by application of a recently described liquid chromatography–mass spectrometry method (19). After addition of the internal standard solution (13C6-arginine and homoarginine), 250 μl of plasma was deproteinized by the addition of 0.5 ml of acetonitrile, the supernatant was evaporated to dryness, and the residue was redissolved in formate buffer. The samples were automatically derivatized with orthophthaldialdehyde/2-mercaptoethanol reagent and were analytically separated on a Merck Superspher RP-18 250 × 4 mm HPLC column, applying a formate buffer/methanol gradient. The analytes were sufficiently separated and selectively detected by a ThermoFinnigan LCQ mass spectrometer equipped with an ESI ion source. The method was validated according to the guidelines for biochemical assays; the coefficient of variation was 7.5% (20). Plasma insulin concentrations were measured immunoenzymatically using an ELISA with monoclonal insulin antibodies, and PTH was measured with an immunoradiometric assay. All other measurements, including high-sensitivity C-reactive protein, were performed using routine laboratory tests and certified methods. Insulin sensitivity was quantified using homeostasis model assessment of insulin resistance: [plasma insulin (mU/L) × plasma glucose (mg/dl) − 405] (21).

Statistical Analyses

Statistical analysis was performed with SPSS for Windows 12.01. Continuous variables were compared between groups with unpaired *t* test or the nonparametric Wilcoxon rank sum test as appropriate. Dichotomized variables were compared using Pearson χ^2 test. The null hypothesis was rejected at $P < 0.05$. Data are presented as mean ± SD. Univariate correlation was performed by Spearman correlation analysis. Furthermore, multivariable adjusted risk estimates for progression end points were calculated using multiple Cox proportional hazards regression analysis. A forward likelihood ratio procedure was used to identify variables associated with progression. Kaplan-Meier time-to-event curves were generated for patients with plasma ADMA concentrations above and below the median value (0.44 μmol/L).

Results

ADMA in Renal Patients

Baseline clinical characteristics and laboratory data of renal patients are reported in Table 1. In renal patients, plasma ADMA levels were significantly correlated with serum creatinine ($r = 0.595$), GFR ($r = -0.591$), PTH ($r = 0.586$), hemoglobin ($r = -0.336$), age ($r = 0.281$), proteinuria ($r = 0.184$), and uric acid ($r = 0.177$; all $P < 0.01$). To elucidate further the relationship between GFR and ADMA blood levels, we stratified renal patients into four groups according to National Kidney Foundation criteria for renal failure: Normal GFR (≥90 ml/min per 1.73 m^2), mild reduction of GFR (≥60 to 89 ml/min per 1.73 m^2), moderate reduction of GFR (≥30 to 59 ml/min per 1.73 m^2), and severe reduction of GFR (≤29 ml/min per 1.73 m^2). Mean plasma

Table 1. Baseline clinical and laboratory data in 227 patients with kidney diseases[a]

Gender (male/female)	154/73 (68%/32%)
Age (yr)	45.7 ± 12.6
Body mass index (kg/m^2)	25.2 ± 3.8
Current smoker (*n*)	49 (22%)
Past smoker (*n*)	57 (25%)
Serum creatinine (mg/dl)	2.02 ± 1.16
GFR (ml/min per 1.73 m^2)	70 ± 42
Proteinuria (g/d per 1.73 m^2)	0.92 ± 0.90
Systolic BP (mmHg)	137 ± 21
Diastolic BP (mmHg)	87 ± 14
Pulse pressure (mmHg)	51 ± 15
Hemoglobin (g/dl)	13.8 ± 2.0
Intact PTH (pmol/L)	11.2 ± 13.7
Insulin (mU/L)	13.9 ± 9.6
Glucose (mg/dl)	98 ± 17
HOMA-IR index	3.59 ± 3.55
Total cholesterol (mg/dl)	215 ± 45
Uric acid (mg/dl)	6.8 ± 1.6
hsCRP (mg/L)	2.65 ± 2.97
ADMA (μmol/L)	0.46 ± 0.12
SDMA (μmol/L)	0.91 ± 0.61

[a]PTH, parathyroid hormone; HOMA-IR, homeostasis model assessment of insulin resistance; hsCRP, High-sensitivity C-reactive protein; ADMA, asymmetric dimethylarginine; SDMA, symmetric dimethylarginine.

ADMA levels were not significantly different in renal patients with normal GFR and mild reduction of GFR but were significantly higher with more advanced stages of renal failure (Figure 1). Plasma SDMA concentrations correlated highly significantly with both GFR ($r = -0.837$, $P < 0.01$) and serum creatinine ($r = 0.894$, $P < 0.01$). In fact, the correlation coefficient for SDMA and GFR was almost identical to that of serum creatinine and GFR ($r = -0.847$; $P < 0.01$). Because of this intimate relationship, we did not use the biologically inactive SDMA in the regression model to rule out multi co-linearity.

Progression End Points during the Prospective Observation Period

A total of 177 (78%) patients of the baseline cohort could be assessed during the follow-up period. Patients who were lost to follow-up had moved away or were not referred by their physicians for follow-up controls to the renal units. Five patients died during the follow-up period, none of them as a result of a fatal cardiovascular event. In addition, 10 patients experienced a nonfatal cardiovascular event during follow-up. The median follow-up time was 54 mo (range 1–82), and during this period, 65 patients reached a progression end point. Table 2 summarizes data in patients with and without progression during the follow-up period. Patients who progressed were significantly older at baseline and had higher serum creatinine, protein excretion rate, and PTH as well as lower GFR and hemoglobin. In addition, they had approximately 30% higher plasma ADMA levels (Figure 2) and more than two times higher plasma SDMA levels. We found no difference in systolic and diastolic BP, but significantly more patients who reached a renal end point during follow-up needed antihypertensive therapy (61 of 65 [94%] *versus* 82 of 112 [73%]; $P < 0.01$). There was no difference in ACE inhibitor treatment between the groups (41 of 65 [63%] *versus* 58 of 112 [52%]; NS).

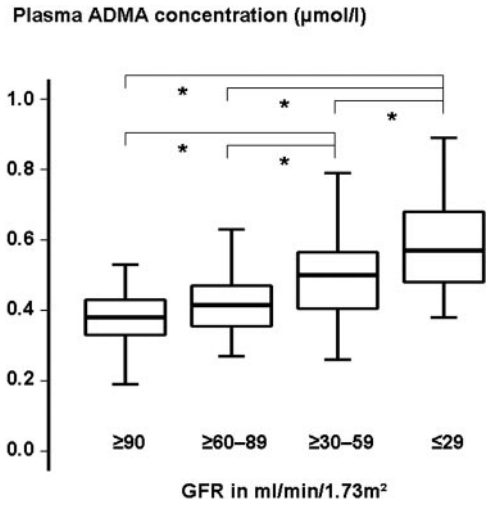

Figure 1. Box plots of plasma asymmetric dimethylarginine (ADMA) concentrations in renal patients with GFR ≥90 ml/min per 1.73 m^2 ($n = 73$; GFR 139 ± 26 ml/min per 1.73 m^2; ADMA 0.37 ± 0.07 µmol/L), GFR ≥60 to 89 ml/min per 1.73 m^2 ($n = 48$; GFR 76 ± 8 ml/min per 1.73 m^2; ADMA 0.43 ± 0.10 µmol/L), GFR ≥30 to 59 ml/min per 1.73 m^2 ($n = 68$; GFR 49 ± 5 ml/min per 1.73 m^2; ADMA 0.46 ± 0.10 µmol/L), and GFR ≤29 ml/min per 1.73 m^2 ($n = 38$; GFR 18 ± 7 ml/min per 1.73 m^2; ADMA 0.58 ± 0.12 µmol/L). Mean plasma ADMA concentration were higher, particularly in patients with advanced renal dysfunction. *$P < 0.01$.

ADMA and Progression

To identify risk factors that were associated with progression over time, we performed a Cox regression analysis using variables that were significantly different in patients who progressed to an end point during follow-up (Table 2). Cox regression analysis revealed baseline serum creatinine (odds ratio 2.00; 95% confidence interval [CI] 1.61 to 2.49; $P < 0.001$) and ADMA (odds ratio 1.47; 95% CI 1.12 to 1.93 for an increment of 0.1 µmol/L; $P < 0.006$) as significant independent predictors of disease progression. Kaplan-Meier curves in kidney patients with plasma ADMA concentrations above (≥0.44 µmol/L) and below the median are presented in Figure 3. In patients with ADMA levels above median, progression was significantly faster ($P < 0.0001$), and their mean follow-up time to a progression end point was 52.8 mo (95% CI 46.9 to 58.8) as compared with 71.6 mo (95% CI 66.2 to 76.9) in patients with ADMA levels below the median.

Discussion

The results of this prospective study from a sizable cohort of white patients with nondiabetic kidney disease point to ADMA as a novel risk marker or even risk factor in the progression of renal disease. The interpretation as a risk factor would be plausible in view of the known role of NO bioavailability in progression. Remarkably, apart from baseline serum creatinine, plasma ADMA was the only independent predictor of progression. The role of increased plasma ADMA concentrations in endothelial/vascular dysfunction and atherosclerosis has been studied in various clinical conditions (9–11). We and others demonstrated recently that at plasma ADMA levels encountered in these conditions, ADMA inhibits NO production, impairs cardiac function, and increases peripheral vascular resistance as well as BP in healthy individuals (22,23). In addition, administration of ADMA caused a long-lasting decrease in renal perfusion and sodium retention even at doses that failed to alter BP (22,24). Thus, ADMA is a biologically active NOS inhibitor with a long duration of action. We hypothesize that chronically elevated ADMA blood levels may promote progression of renal (vascular) disease *via* endothelial damage as a consequence of reduced NO availability (4–6). This assumption is also supported by the observation that ADMA is a significant determinant of the age-related increase in renovascular resistance and decrease in renal perfusion (25).

In their seminal paper, Vallance *et al.* (26) reported markedly increased plasma concentrations of ADMA in patients who were on maintenance hemodialysis. They hypothesized that the high incidence of hypertension and atherosclerosis encountered in patients with terminal renal failure might be caused, at least in part, by reduced NO bioavailability secondary to accumulation of ADMA as a result of reduced renal excretion. Indeed, in several subsequent studies, significantly increased plasma ADMA levels have been documented in patients with

Table 2. Clinical and laboratory data of renal patients with and without progression to a renal end point during the follow-up period

	Nonprogressors (n = 112)	Progressors (n = 65)
Gender (male/female)	74/38 (66%/34%)	44/21 (68%/32%)
Age (yr)	44.8 ± 12.6	49.1 ± 11.0[a]
Body mass index (kg/m²)	24.9 ± 3.5	25.7 ± 3.9
Current smoker (n)	18 (16%)	16 (25%)
Past smoker (n)	26 (23%)	18 (28%)
Serum creatinine (mg/dl)	1.54 ± 0.61	3.21 ± 1.31[b]
GFR (ml/min per 1.73 m²)	79 ± 38	38 ± 25[b]
Proteinuria (g/d per 1.73 m²)	0.87 ± 0.95	1.25 ± 0.83[b]
Systolic BP (mmHg)	136 ± 22	137 ± 17
Diastolic BP (mmHg)	86 ± 14	88 ± 12
Pulse pressure (mmHg)	50 ± 16	50 ± 14
Hemoglobin (g/dl)	14.2 ± 1.5	12.6 ± 1.9[b]
Intact PTH (pmol/L)	6.5 ± 5.3	22.5 ± 20.0[b]
Insulin (mU/L)	13.7 ± 11.1	13.1 ± 6.6
Glucose (mg/dl)	99 ± 16	97 ± 14
HOMA-IR	3.58 ± 4.15	3.25 ± 2.03
Total cholesterol (mg/dl)	215 ± 42	217 ± 46
Uric acid (mg/dl)	6.6 ± 1.6	7.0 ± 1.6
hsCRP (mg/L)	2.78 ± 3.18	2.89 ± 3.10
ADMA (μmol/l)	0.42 ± 0.09	0.55 ± 0.11[b]
SDMA (μmol/l)	0.68 ± 0.37	1.46 ± 0.67[b]

[a] $P < 0.05$.
[b] $P < 0.01$.

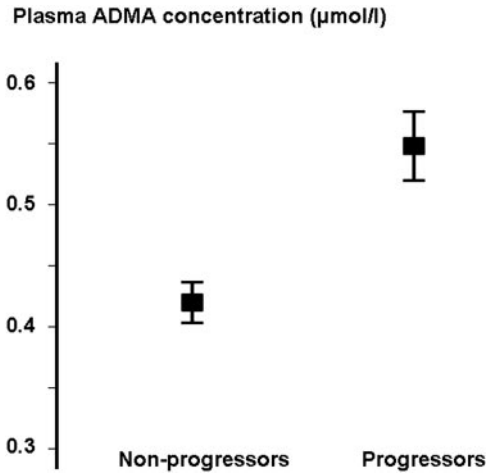

Figure 2. Plasma ADMA concentrations in renal patients who reached a progression end point (n = 65) and in patients who did not progress (n = 112) during follow-up. Mean plasma ADMA concentration was significantly higher ($P < 0.01$) in patients who doubled their serum creatinine and/or reached terminal renal failure necessitating renal replacement therapy. Data are presented as 95% confidence intervals of the mean.

terminal renal failure (27–29). It has also been shown that these ADMA concentrations are sufficiently high to reduce significantly NO production *ex vivo* (30). The results of Zoccali *et al.* (29) in a prospective study that comprised 225 patients with terminal renal failure are also in line with an important pathophysiologic role of ADMA in (cardio)vascular dysfunction. In this study, increased plasma ADMA concentrations not only were significantly related to the severity of carotid atherosclerosis but also were the second strongest predictor (after age) of cardiovascular mortality among several traditional and nontraditional risk factors assessed (29,31).

Results of recent studies question the role of renal excretion (*i.e.*, filtration) as the main route of ADMA elimination. Rather, reduced activity of dimethylarginine dimethylaminohydrolase (DDAH; the enzyme that hydrolyzes ADMA to dimethylamine and L-citrullin had been proposed (9–11,13,27,32). It has been estimated that in humans, approximately 300 μmol of ADMA is generated per day, approximately 250 μmol of which is metabolized by DDAH, whereas only a minor amount is excreted unchanged by the kidneys (23). The finding that DDAH and NOS are co-localized in endothelial cells within the glomerulus and in renal tubular cells supports the hypothesis that the intracellular ADMA concentration is actively regulated in NO-generating endothelial cells within the kidney as well (14). Thus, destruction of DDAH-rich renal tissue can impair ADMA degradation. Indirect proof for this assumption comes from metabolic balance studies in individuals with normal renal function, which have revealed that the kidney is a major extraction site for ADMA from the circulation (15). Collectively, these data may explain why even minor renal dysfunction inexorably leads to accumulation of ADMA. Moreover, modulation of DDAH activity to lower plasma ADMA levels may represent a novel therapeutic target to retard progression. In this respect, inhibition of the renin-angiotensin system may not be enough, because we could not find a significant effect of chronic angiotensin II receptor blockade on plasma ADMA concentrations in a recent double-blind, placebo-controlled,

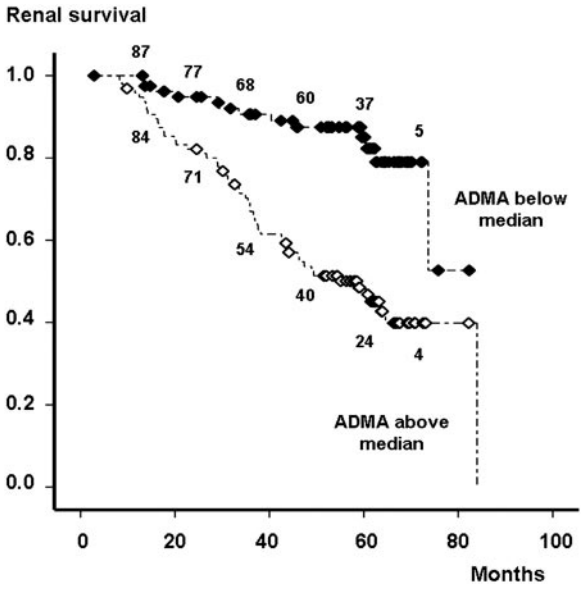

Figure 3. Kaplan-Meier curves of renal end points in patients with infra- and supramedian plasma ADMA concentrations. In patients with supramedian plasma ADMA concentrations ($\geq$0.44 μmol/L), progression was significantly faster ($P < 0.0001$). Numbers near the survival curves represent the number of patients who were at risk in each group at the follow-up times 12, 24, 36, 48, 60, and 72 mo.

randomized study (33). Thus, other treatment strategies to reduce ADMA in patients with kidney diseases should be evaluated. In contrast to ADMA, its biologically inactive stereoisomer SDMA is not metabolized by (renal) DDAH. We and others have previously shown that plasma SDMA levels are closely related to renal function because SDMA is thought to be filtered exclusively by the kidney (13,26,27). In our study, we also found a very close relationship between serum creatinine and SDMA ($r = 0.894$), and we speculate that SDMA is equal to serum creatinine as a marker of renal function.

Our finding of the striking predictive power of baseline serum creatinine as a progression marker highlights the importance of impaired renal function as a key determinant of progressive renal damage. The odds ratio for progression was even higher for creatinine than for ADMA. In other words, once renal function is impaired, it will inexorably perpetuate further progression. In this respect, we have to point out that our cohort studied comprised relatively young patients, and most of them had mild to moderate impairment of renal function or even normal renal function at the baseline examination. An important target for arresting progressive renal disease is treatment of high BP, preferably with inhibitors of the renin-angiotensin system (34,35). In our cohort, systolic and diastolic BP at baseline were comparable in patients who progressed to a renal end point during follow-up and in those who did not. The former needed more aggressive antihypertensive treatment to achieve the same level of BP control, whereas the use of ACE inhibitors was comparable between groups. BP control was identical (and acceptable) in patients with and without significant progression. This may have permitted identification of further progression promoters such as ADMA.

In conclusion, ADMA, an endogenous NO synthase inhibitor, is significantly associated with progression in patients with nondiabetic mild to moderate kidney disease. Lowering plasma ADMA concentrations therefore may represent a novel therapeutic target for prevention of progressive renal damage.

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See related editorial, "Asymmetric Dimethylargine and Kidney Disease—Marker or Mediator?," on pages 2254–2256.