ADMA Levels Correlate with Proteinuria, Secondary Amyloidosis, and Endothelial Dysfunction

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ABSTRACT

Asymmetric dimethyl-arginine (ADMA), a residue of the proteolysis of arginine-methylated proteins, is a potent inhibitor of nitric oxide synthesis. The increased protein turnover that accompanies proteinuric secondary amyloidosis may increase circulating levels of ADMA, and this may contribute to endothelial dysfunction. We performed a cross-sectional study of 121 nondiabetic proteinuric patients with normal GFR (including 39 patients with nephrotic-range proteinuria and secondary amyloidosis) and 50 age-, sex-, and BMI–matched healthy controls. The proteinuric patients had higher levels of serum ADMA, symmetric dimethyl-arginine (SDMA), high-sensitivity C-reactive protein (hsCRP), and insulin resistance (homeostasis model assessment index) than controls. Compared with controls, brachial artery flow-mediated dilatation (FMD), serum L-Arginine, and the L-Arginine/ADMA ratio were significantly lower among proteinuric patients, suggesting greater endothelial dysfunction. When patients with secondary amyloidosis were compared with patients with glomerulonephritis who had similar levels of proteinuria, those with amyloidosis had higher ADMA and SDMA levels and lower L-Arginine/ADMA ratios and FMD measurements (P < 0.001 for all). Finally, even after adjusting for confounders, ADMA level correlated with both proteinuria and the presence of secondary amyloidosis, and was an independent predictor of FMD. We propose that ADMA synthesis may be increased in chronic kidney disease, especially in secondary amyloidosis, and this may explain part of the mechanism by which proteinuria increases cardiovascular morbidity and mortality.


Secondary amyloidosis (SA), also called reactive amyloidosis or amyloidosis AA, occurs in patients with chronic inflammatory diseases because of the extracellular deposition of normally soluble autologous protein in a characteristic abnormal fibrillar form.1 Familial Mediterranean fever (FMF) is an autosomal recessive autoimmune disorder common in Mediterranean countries such as Turkey and is associated with the development of SA.2,3 Renal involvement is very common in FMF-related SA, which is thus a prevalent cause of both microand macroproteinuria and chronic kidney disease (CKD) in this geographic region.2–4

Regardless of primary cause, patients with CKD as a result of SA have an even poorer outcome than other CKD patients.5–7 Although the exact causes for this is are not known, it has been suggested that SA itself is a cause of cardiovascular disease (CVD).8 ADMA is a residue of proteolysis of arginine-methylated pro-

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Table 1. Etiology of glomerular dysfunction and basic demographics in proteinuric patients and controls

<table>
<thead>
<tr>
<th>Variables</th>
<th>Patients (n = 121)</th>
<th>Healthy, Age and Sex-Matched Controls (n = 50)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nonnephrotic Proteinuria (&lt;3.5 g/d) (n = 43)</td>
<td>Nephrotic Range Proteinuria without Amyloidosis (&gt;3.5 g/d) (n = 39)</td>
</tr>
<tr>
<td>Age, yr</td>
<td>31 ± 5</td>
<td>31 ± 6</td>
</tr>
<tr>
<td>Sex (male/female)</td>
<td>22/21</td>
<td>20/19</td>
</tr>
<tr>
<td>Biopsy-verified glomerular disease, n</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FSGS</td>
<td>19</td>
<td>10</td>
</tr>
<tr>
<td>IgA nephropathy</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>secondary FSGS</td>
<td>3</td>
<td>—</td>
</tr>
<tr>
<td>membranous nephropathy</td>
<td>11</td>
<td>1</td>
</tr>
<tr>
<td>MPGN</td>
<td>2</td>
<td>—</td>
</tr>
<tr>
<td>minimal mesangial proliferation</td>
<td>2</td>
<td>—</td>
</tr>
<tr>
<td>lupus nephritis</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>minimal change disease</td>
<td>—</td>
<td>19</td>
</tr>
<tr>
<td>amyloidosis</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

*aFSGS, focal segmental glomerulosclerosis; MPGN, membranoproliferative glomerulonephritis.

Figure 1. (A) Serum ADMA (ANOVA, P < 0.001), (B) SDMA (ANOVA, P < 0.001), (C) FMD (ANOVA, P < 0.001), and (D) HOMA (ANOVA, P < 0.001) levels in 50 control subjects and patients with nonnephrotic-range proteinuria (n = 43), nephrotic range proteinuria (n = 39), or amyloidosis and nephritic range proteinuria (n = 39).
teins, and it is a potent inhibitor of nitric oxide synthesis. It is cleared by the kidney in vivo, and is found in elevated circulating levels in CKD. We recently reported that nondiabetic proteinuria is associated with elevation of ADMA in CKD. In this study, we hypothesized that this might be the result of an abnormal protein turnover in proteinuria, which might be further exacerbated in SA. We furthermore hypothesized that an ADMA-associated endothelial dysfunction would be yet another factor linking proteinuric patients, distributed as peritonitis (n = 32, 82%), fever (n = 29, 74%), arthritis (n = 9, 23%), pleuritis (n = 3, 8%), myalgia (n = 8, 21%), appendectomy with noninfected appendix (n = 9, 23%), and erysipelas-like erythema (n = 3, 8%).
significant differences between groups, are shown in Table 2. Briefly, patients with nephrotic-range proteinuria had significantly higher ADMA and SDMA concentrations than healthy subjects or patients with nonnephrotic proteinuria, whereas the L-Arginine/ADMA ratio was significantly lower.

Although we found no significant differences between the two groups with nephrotic-range proteinuria in age, sex, BMI, eGFR, BP, amount of proteinuria, L-arginine, hsCRP, serum albumin, cholesterol, or triglycerides, significant differences between these two groups in HOMA, FMD, serum SDMA, ADMA levels, ADMA/SDMA ratio, and L-Arginine/ADMA ratio were observed (Figure 1).

Table 3. Spearman Rank correlations between investigated variables and proteinuria and endothelial function (FMD) in all proteinuric patients and in the separate groups

<table>
<thead>
<tr>
<th></th>
<th>All Proteinuric Patients (n = 121)</th>
<th>Nonnephrotic Proteinuria (&lt;3.5 g/d) (n = 43)</th>
<th>Nephrotic-Range Proteinuria without Amyloidosis (&gt;3.5 g/d) (n = 39)</th>
<th>Nephrotic-Range Proteinuria with Amyloidosis (&gt;3.5 g/d) (n = 39)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteinuria (mg/d)</td>
<td>FMD (%)</td>
<td>Proteinuria (mg/d)</td>
<td>FMD (%)</td>
<td>Proteinuria (mg/d)</td>
</tr>
<tr>
<td>Age, yr</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>GFR, ml/min per 1.73 m²</td>
<td>0.27a</td>
<td>0.60b</td>
<td>−0.26a</td>
<td>−0.43b</td>
</tr>
<tr>
<td>Serum albumin, g/d</td>
<td>−0.60b</td>
<td>0.48b</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>hsCRP, mg/L</td>
<td>0.47b</td>
<td>−0.40b</td>
<td>0.53b</td>
<td>NS</td>
</tr>
<tr>
<td>Triglycerides, mg/dl</td>
<td>0.64b</td>
<td>−0.38b</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Total Cholesterol, mg/dl</td>
<td>0.57a</td>
<td>−0.25a</td>
<td>0.33a</td>
<td>NS</td>
</tr>
<tr>
<td>LDL Cholesterol, mg/dl</td>
<td>0.60b</td>
<td>−0.38b</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Proteinuria, g/d</td>
<td>NA</td>
<td>−0.55b</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>FMD, %</td>
<td>−0.55b</td>
<td>NA</td>
<td>NS</td>
<td>NA</td>
</tr>
<tr>
<td>L-Arginine, µmol/L</td>
<td>−0.27a</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>L-Arginine/ADMA</td>
<td>−0.88b</td>
<td>0.59b</td>
<td>−0.68b</td>
<td>NS</td>
</tr>
<tr>
<td>SDMA, µmol/L</td>
<td>0.85b</td>
<td>−0.58b</td>
<td>0.72b</td>
<td>NS</td>
</tr>
<tr>
<td>ADMA, µmol/L</td>
<td>0.88b</td>
<td>−0.60b</td>
<td>0.83b</td>
<td>NS</td>
</tr>
</tbody>
</table>

NA, not applicable; NS, not significant. *P < 0.05, **P < 0.001.

Univariate Correlations

Significant correlations between investigated variables are given in Table 3. Briefly, when all patients were grouped together, significant positive correlations were observed between the level of proteinuria and ADMA, SDMA, L-Arginine/ADMA ratio, and hsCRP, respectively. FMD levels correlated negatively with proteinuria, ADMA, SDMA, L-Arginine/ADMA ratio, hsCRP, and HOMA. All these differences remained significant when the subgroup of proteinuric SA patients was studied separately. In proteinuric glomerulonephritis patients, the associations remained between proteinuria and ADMA, SDMA, L-Arginine/ADMA ratio, and hsCRP (data not shown).
Multivariate Regression Analysis
To test the relative strength of the observed correlations, we performed multivariate regression analysis, incorporating all factors significantly associated with FMD and ADMA/L-Arginine ratio in univariate analysis, as well as sex and age (Table 4). In the first model (adjusted $r^2 = 0.34$), ADMA levels ($>2.76 \mu\text{mol/L}$; $r^2 = 0.27$, $P = 0.0002$), serum albumin ($>3.5 \text{g/dL}$; $r^2 = 0.21$, $P = 0.004$), and HOMA ($>2.1$; $r^2 = 0.10$, $P = 0.01$) were found to be independently related to FMD levels. In the second model (adjusted $r^2 = 0.87$), proteinuria ($>2.5 \text{g/dL}$; $r^2 = 0.40$, $P < 0.001$), amyloidosis (yes/no; $r^2 = 0.42$, $P = 0.0003$), and SDMA ($>3.16 \mu\text{mol/L}$; $r^2 = 0.70$, $P < 0.001$) were found to be related to L-Arginine/ADMA levels.

DISCUSSION
In this study, we investigated predictors of endothelial dysfunction assessed by FMD in 121 proteinuric patients with normal GFR and in 50 healthy controls. For what we believe to be the first time, we include a large ($n = 39$) group of patients with nephrotic-range proteinuria caused by SA, and we show that these patients have a significantly worse endothelial function despite a similar degree of proteinuria, a comparable metabolic profile, and identical level of inflammation. Because FMD in this study was independently associated with both circulating methylarginines and proteinuria, we hypothesize that endothelial dysfunction resulting from increased methylarginine concentrations may be one factor contributing to the elevated risk of CVD in proteinuric patients, especially in those with amyloidosis.

In the general population, ADMA levels or L-Arginine/ADMA ratios are independently associated with endothelial dysfunction and have been reported to be elevated in patients with CVD. Because the kidneys provide a significant route for clearance of methylarginines, it is perhaps not surprising that ADMA and SDMA concentrations increase in CKD patients even without proteinuria, or that proteinuria with reduced renal function is associated with both elevated ADMA and endothelial dysfunction. However, the patients in our study all had a nearly normal renal function. Inflammation, defined here as an elevated serum hsCRP, is also known to influence ADMA and has been proposed to be one factor linking ADMA to CVD. In agreement with this, renal patients with nonnephrotic-range proteinuria in our study had both lower CRP and ADMA. Another possible link between ADMA and CVD is the reported association with insulin resistance. However, the present data do not show any independent association between ADMA and either HOMA or eGFR in any of the studied groups. This could suggest that, although renal parenchymal damage may indeed contribute to increase ADMA through altered insulin signaling in our particular population, other factors were more important or could be more accurately measured. Of interest for future studies, in this study ADMA and SDMA were both elevated most in the amyloidosis patients with marked proteinuria (Table 2), supporting a similar finding in diabetic patients without renal disease. Further, after adjustment for ADMA the significant association between the degree of proteinuria and FMD was lost (Table 4).

In light of these results, we speculate that at least certain types of kidney diseases (e.g., amyloidosis) are associated not only with decreased renal clearance of methylarginines, but also with an increased production—hypothetically caused by an increased turnover of methylated proteins as a result of increased de novo synthesis of albumin and other common circulating proteins lost as a result of glomerular membrane damage as well as putatively through deposition in amyloid plaques. It should be stressed that although our study measured circulating methylarginines, it is not known whether these are biologically active in themselves or just a reflection of high intracellular levels that matter more. Indeed, concern has been expressed that the typical plasma levels found in healthy individuals (0.5 to 1.2 $\mu\text{mol/L}$) are too low to exert biological activity.

Finally, and as earlier reported, this study reconfirms the malignant links between proteinuria and both dysmetabolism (hyperlipidemia and insulin resistance) and endothelial dysfunction (assessed by FMD). Whereas de novo protein synthesis by the liver has been suggested to explain the rapid increase in cholesterol seen after the onset of proteinuria, we show that proteinuria is also associated with increased ADMA formation, which in turn is thought to be an important inducer of insulin resistance.

Several limitations of our study should be discussed. Foremost, the cross-sectional nature of the study prevents any conclusion about causality. Furthermore, the lack of measurement of de novo protein synthesis and dimethylarginine dimethylaminohydrolase levels limits our understanding about the mechanisms involved. Finally, we cannot exclude that ADMA/SDMA elevation is simply an epiphenomenon associated with general dysmetabolism in the proteinuric state, perhaps the result of increased protein catabolism linked to insulin resistance. Clearly, mechanistic research is warranted to further illuminate the hypothetical links between protein turnover, methylarginines, and endothelial function and dysmetabolism in proteinuric CKD.

To conclude, we confirm that proteinuria is associated with significant endothelial dysfunction, dysmetabolism, and inflammation even in the absence of overt renal failure. Additionally, we report that the degree of proteinuria and the presence of amyloidosis as its cause additively and independently influence circulating levels of ADMA/SDMA and FMD.

CONCISE METHODS
Patients
Patients were recruited from first referrals to the renal outpatient clinic of the Gülhane School of Medicine in Ankara, Turkey, between September 2003 and October 2006. The local ethical committee of the Gülhane School of Medicine approved the study protocol, and informed consent was obtained from each subject. Among a referred population of 572 patients without established renal disease, 253 patients had isolated proteinuria (24 h protein excretion $>500 \text{mg/d}$ with normal GFR). From these latter patients, we enrolled 121 pa-
tients in a cross-sectional study. Exclusion criteria were untreated hypertension according to the Joint National Committee VII criteria and/or the current use of antihypertensive medications (n = 63), overt diabetes mellitus (n = 91), obesity (BMI > 30 kg/m²; n = 32), clinical CVD (defined as the presence or history of ischemic heart disease, peripheral vascular disease and/or a cerebrovascular event; n = 44), and patients previously treated with immunosuppressive drugs for proteinuria (n = 23). Among patients with amyloidosis, we also excluded those without nephrotic-range proteinuria (n = 17) and/or abnormal renal function (eGFR < 70 ml/min; n = 9). In all, 121 patients were eligible for inclusion in the study (61 men; mean age, 31 ± 5 yr; all white). Ninety-six patients were first referrals, and in these patients the duration of proteinuria after initial diagnosis was not known. Kidney biopsy was performed in 96 patients on admission, whereas 25 patients had their biopsy performed before referral. Biopsies of all patients classified as SA stained positive with Congo red dye. According to the renal biopsy results and standard histological criteria, all recruited patients were assigned to one of the two groups, SA and primary glomerulopathy, respectively. Most of the patients were already participating in an ongoing prospective study, and part of this patient material has thus been previously described.

All enrolled subjects were evaluated by standard physical examination, chest x-ray, baseline electrocardiogram, two-dimensional echocardiography, and routine biochemical laboratory tests, including liver and kidney function tests and 24-h urinary protein measurements. FMD and venous blood samples were taken after a 2-wk washout period, during which time no vasoactive drugs (including colchicines) were given.

The study also recruited a control group comprising 50 healthy, unrelated subjects matched for age, sex, and BMI by advertisement in the hospital (25 men; mean age, 31 ± 5 yr). The controls first underwent comprehensive physical and laboratory evaluation to ascertain that they had no hypertension, metabolic, hepatic, or renal diseases. Control patients were also required to have no family history of hypertension or diabetes mellitus and underwent the same clinical and biochemical evaluations as the patients.

Laboratory and Imaging Procedures
Arterial BP was measured three times in the morning after a 15-min resting period and mean values were calculated. Blood sampling was done after an overnight fast. Routine biochemical methods were used throughout. To increase the precision of proteinuria estimates, 24-hr urine collection was performed three times and the average proteinuria used. The primary glomerulopathy group was further divided according to the result of proteinuria measurements into those with nephrotic-range proteinuria (urinary protein ≥ 3.5 g; n = 43) and those without (urinary protein < 3.5 g; n = 39). Blood urea, serum creatinine, as well as fasting plasma glucose, total protein, serum albumin, total cholesterol, HDL cholesterol, and triglycerides were determined with enzymatic colorimetry on an Olympus AU 600 autoanalyzer using reagents from Olympus Diagnostics GmbH (Hamburg, Germany). LDL cholesterol was calculated with Friedewald’s formula. Twenty-four-hour proteinuria was determined by a turbidimetric test using TCA. The serum basal insulin value was determined using a coated tube method (DPC, Inc., Los Angeles, CA).

Renal function was estimated by the modified Modification of Diet in Renal Disease (MDRD) formula in ml/min and expressed per 1.73 m² of body surface area. HOMA was then computed as: HOMA insulin resistance (HOMA-IR) = fasting plasma glucose (mg/dl) × immunoreactive insulin (µIU/ml)/405. Serum hsCRP was determined by turbidimetric fixed rate method by an automated analyzer (Olympus AU-2700, Chimiza, Japan).

Measurements of ADMA, SDMA, and L-Arginine
Measurements of serum ADMA, SDMA, and L-Arginine were made using HPLC as described by Chen et al. In brief, 20 mg 5-sulfosalicylic acid (5-SSA) was added to 1 ml serum and the mixture was left in an ice-bath for 10 min. The precipitated protein was removed by centrifugation at 2000 g for 10 min. Ten microliters of the supernatant, which was filtered through a 0.2-µm filter, was mixed with 100 µl of derivatization reagent (prepared by dissolving 10 mg o-phthalaldehyde in 0.5 ml methanol, 2 ml 0.4 M borate buffer (pH 10.0), and 30 µl 2-mercaptoethanol) and then injected into the chromatographic system. Separation of ADMA was achieved with a 150 × 4 mm I.D. Nova-pak C18 column with a particle size of 5 µm (Waters, Millipore, Milford, MA) using 50 mM sodium acetate (pH 6.8), methanol, and tetrahydrofuran as mobile phase (A, 82:17:1; B, 22:77:1) at a flow-rate of 1.0 ml/min. The areas of peaks detected by the fluorescence detector (Ex: 338 nm; Em: 425nm) were used as quantification. The variability of the method was <7%, and the detection limit of the assay was 0.01 µM.

Ultrasonic Measurements
In all patients and controls, we determined endothelial dysfunction according to Celermajer et al. Measurements were made by a single observer using an ATL 5000 ultrasound system (Advanced Technology Laboratories, Inc., Bothell, WA) with a 12-Mhz probe. The subjects remained at rest in the supine position for at least 15 min before the examination started. The subject’s arm was comfortably immobilized in the extended position to allow consistent recording of the brachial artery 2 to 4 cm above the antecubital fossa. Three adjacent measurements of end-diastolic brachial artery diameter were made from single two-dimensional frames. All ultrasound images were recorded on S-VHS videotape for subsequent blinded analysis. A pneumatic tourniquet was then inflated to 200 mmHg with obliteration of the radial pulse. After 5 min the cuff was deflated. Flow measurements were made 60 seconds after deflation. The maximum FMD (FMDmax) diameters were calculated as the average of three consecutive maximum diameter measurements. The percent change in average FMDmax diameter compared with baseline resting diameter was defined as FMD. The intraobserver coefficient of variation for FMD was 5.1%.

Statistical Analyses
Nonnormally distributed variables were expressed as median (range), and normally distributed variables were expressed as mean ± SD as appropriate. A P value <0.05 was considered to be statistically significant. Between-group comparisons were performed for nominal variables using the χ² test. Differences between patients and controls groups were tested for significance using the ANOVA followed by post hoc adjusted Tukey-Kramer test for multiple comparisons. Spear-
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DISCLOSURES

Dr. Lindholm is an employee of Baxter Healthcare, Inc. Dr. Stenvinkel is a member of the scientific advisory board of Gambro AB.

REFERENCES


