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Asymmetric Dimethylarginine, C-Reactive Protein, and Carotid Intima-Media Thickness in End-Stage Renal Disease

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Abstract. Asymmetric dimethylarginine (ADMA) is an endogenous inhibitor of nitric oxide (NO) synthase that has been linked to endothelial dysfunction and atherosclerosis in the general population. ADMA is also elevated in end-stage renal disease and may contribute to the high cardiovascular risk in patients with chronic renal failure. A prospective cohort study was performed to investigate the relationship between plasma ADMA, C-reactive protein (CRP), and intima-media thickness (IMT) in 90 patients undergoing hemodialysis. In the baseline study, plasma ADMA was directly related to IMT both on univariate analysis ($r = 0.32$, $P = 0.002$) and on multiple regression analysis ($\beta = 0.23$, $P = 0.01$). In the follow-up study (15 mo) IMT changes were significantly related to ADMA ($r = 0.51$, $P = 0.02$) and serum CRP ($r = 0.53$, $P = 0.01$) in patients with initially normal IMT. In these patients, ADMA and CRP were strongly interrelated ($r = 0.64$, $P = 0.002$), and on multiple regression analysis the interaction between ADMA and CRP emerged as the sole independent predictor of the progression of intimal lesions. Independently of other risk factors, plasma ADMA in patients on hemodialysis is significantly related to IMT. Furthermore, in patients with initially normal IMT, ADMA and CRP are interacting factors in the progression of carotid intimal lesions. These data support the hypothesis that accumulation of this endogenous inhibitor of NO synthase is an important risk factor for cardiovascular disease in chronic renal failure and suggest a possible link between ADMA and inflammation.

Several lines of evidence implicate the endogenous inhibitor of nitric oxide (NO) synthase, asymmetric dimethylarginine (ADMA), in human atherosclerosis (1). In young subjects with hypercholesterolemia, elevation of ADMA is associated with impaired endothelium-dependent vasodilation, an abnormality that is reversed by administration of L-arginine (2). Related to these observations in humans are data in hypercholesterolemic rabbits that have shown elevated plasma ADMA concentrations in these animals (3). Furthermore, in cultured human endothelial cells, this substance increases oxidative stress and potentiates monocyte binding, which are two key processes in the genesis and evolution of atherosclerosis (4). These observations suggest that the thickening of the intima-media complex recently reported in middle-aged healthy individuals with elevated ADMA levels (5) may be the morphologic expression of the link between ADMA and endothelial dysfunction.

Given findings of such a link in healthy individuals, the putative atherogenic potential of ADMA might be exemplified in patients with end-stage renal disease (ESRD). In patients undergoing dialysis, there is a strong association between cardiovascular remodeling and endothelial dysfunction (6). ADMA is markedly increased in patients with chronic renal failure (7–13), and ADMA levels have been associated with atherosclerotic complications in these patients (10). In a recent prospective cohort study, we found that circulating ADMA is a strong predictor of all-cause and cardiovascular mortality in patients with ESRD (11). Studying the relationship between intima-media thickness (IMT) measured by high definition echo color Doppler and plasma ADMA concentration in patients undergoing dialysis may represent a further test for the hypothesis that ADMA is involved in human atherosclerosis. Elevated C-reactive protein (CRP) is another predictor for morbidity and mortality in renal and cardiovascular disease (14). This acute-phase reactant is widely considered as a marker of underlying inflammatory processes in ESRD (15), and it is associated with carotid artery intima thickness (16). This prospective cohort study offers insight into a possible link among ADMA, CRP, IMT, and IMT changes at follow-up in a sizable group of patients on chronic hemodialysis.

Materials and Methods

The protocol conformed to the ethical guidelines of our Institutions, and informed consent was obtained from each participant. All studies

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were performed on a midweek nondialysis day, in the morning between 8 a.m. and 1 p.m.

Patients

Ninety patients on hemodialysis (51 men and 39 women) with ESRD who had been treated for at least 6 mo (median duration of dialysis treatment, 73 mo; interquartile range, 23 to 151 mo) without clinical evidence of circulatory congestion (defined as dyspnea in addition to two of the following conditions: raised jugular pressure, bibasilar crackles, pulmonary venous hypertension or interstitial edema on chest x-ray requiring hospitalization or extra ultrafiltration, and ejection fraction <35%) were considered eligible for the study. Five patients had had a stroke, 8 myocardial infarction, and 16 peripheral vascular disease. The whole study cohort represented ~70% of the whole dialysis population of the urban area of Reggio Cal.

Patients were being treated three times weekly with standard bicarbonate dialysis (138 mmol/L Na, 35 mmol/L HCO_3, 1.5 mmol/L K, 1.25 mmol/L Ca, and 0.75 mmol/L Mg) that used 1.1- to 1.7-m^2 dialyzers either with cuprophan or semisynthetic membranes. Dry weight was targeted in each case to achieve a normotensive edema-free state. Thirty-nine patients were habitual smokers (21 ± 16 cigarettes/d), and 46 were on antihypertensive treatment (29 on monotherapy with angiotensin-converting enzyme inhibitors, AT-1 antagonists, calcium-channel blockers, or alpha and beta blockers, and 17 on double or triple therapy with various combinations of these drugs). Demographic, anthropometric, clinical, and biochemical parameters of the whole cohort are given in Table 1.

Carotid Ultrasonography Study

In all patients, ultrasonographic studies on common carotid arteries were performed bilaterally by a single observer who was blinded to the clinical and biochemical data. Repeated studies in 105 patients on dialysis by a blinded observer in our laboratory showed that IMT represents a reliable measurement in patients on dialysis (coefficient of variation, 5.5%; mean difference, −0.01 mm). All studies were performed with a Hewlett Packard Sonos 1500 by use of a 7.5-MHz high-resolution probe. IMT was defined as a low-level echo gray band that did not project into the arterial lumen (17) and was measured during end-diastole as the distance from the leading edge of the second echogenic line of the far walls of the common carotid artery on both sides. Measurements were performed at 0.5, 1, and 2 cm below the bifurcation (three measurements on each side), and the average measurement was taken as IMT. In our laboratory, the upper limit of the normal range of IMT is 0.95 mm. This value corresponds to the average value ±3 SD in a group of 70 Italian healthy normotensive volunteers (age 52 ± 14 yr) (18). The number of atherosclerotic plaques (19) (either as faint gray echoes [soft plaques] or bright white

Table 1. Demographic, anthropometric, clinical, biochemical, and echo color Doppler data of the study population[a]

Parameter	Whole Group	Survivors (n = 72)	Nonsurvivors (n = 18)
Somatometric data			
age (yr)	57.0 ± 13.9	54.7 ± 14.0	66.2 ± 9.2*
men/women	51/39	41/31	10/8
BMI (kg/m^2)	24.4 ± 4.5	24.1 ± 4.0	25.7 ± 5.9
Cardiovascular risk factors			
systolic pressure (mmHg)	142.1 ± 22.0	142.2 ± 22.5	141.9 ± 20.8
diastolic pressure (mmHg)	75.1 ± 12.5	75.2 ± 12.8	74.3 ± 11.6
diabetics (%)	11.1	5.6	33.3***
smokers (%)	43.3	44.4	38.9
hypercholesterolemia (%) (serum total cholesterol >5.17 mmol/L)	57.8	58.3	55.6
Biochemical data			
plasma ADMA (μmol/L)	4.22 ± 1.73	3.92 ± 1.50	5.41 ± 2.12**
plasma homocysteine (μmol/L)	27.5 (20.1–46.1)	26.8 (20.0–48.6)	29.3 (21.7–42.6)
serum total cholesterol (mmol/L)	5.55 ± 1.49	5.57 ± 1.51	5.49 ± 1.47
serum triglycerides (mmol/L)	1.96 ± 1.02	1.91 ± 1.02	2.17 ± 1.05
serum calcium (mmol/L)	2.3 ± 0.3	2.3 ± 0.3	2.2 ± 0.4
serum phosphate (mmol/L)	2.0 ± 0.5	2.0 ± 0.6	2.0 ± 0.5
serum CRP (mg/L)	9.6(3.4–19.1)	7.9(3.4–16.0)	23.9(11.8–38.8)***
serum albumin (g/L)	41 ± 6	41 ± 0.6	38 ± 4*
hemoglobin (g/L)	104 ± 20	1.04 ± 18	104 ± 26
Kt/V	1.28 ± 0.29	1.32 ± 0.28	1.13 ± 0.28**
Echo Color Doppler data			
intima-media thickness (mm)	1.06 ± 0.24	1.03 ± 0.24	1.16 ± 0.25*
internal diameter (mm)	6.8 ± 1.0	6.8 ± 1.0	7.0 ± 1.0
number of plaques	2 (1 to 5)	2 (0 to 4)	5 (2 to 9)**

[a] Data are mean ± SD, median (interquartile range), or percentage of frequency, as appropriate. Kt/V denotes fractional urea clearance. ADMA, asymmetric dimethyl arginine; BMI, body mass index; CRP, C-reactive protein. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, survivors *versus* not survivors.

echoes [calcified plaque] protruding into the lumen) detected in the bulbar area (from 2 cm below to 2 cm above the bifurcation) of the carotid arteries was recorded on both sides and summed up. The internal diameter of the common carotid artery (DCCA) was measured bilaterally 2 cm below the bifurcation during end diastole, and the average measurement was taken as DCCA. IMT and DCCA measurements were always performed in plaque-free arterial segments.

Follow-Up Study

After the initial assessment, echo color Doppler was repeated after 15.1 ± 1.1 mo by the same observer, who was again blinded to clinical and laboratory data.

Laboratory Measurements

Blood sampling was performed after 20 to 30 min of quiet resting in semirecumbent position, always 1 to 4 h before carotid ultrasonography. Samples were taken into prechilled ethylenediaminetetraacetate vacutainers, placed immediately on ice, centrifuged within 30 min at 4°C, and the plasma stored at −80°C until analyses.

Serum cholesterol, albumin, calcium, phosphate, and hemoglobin measurements were made by use of standard methods in the routine clinical laboratory. CRP was measured by a commercially available kit (Behring, Scoppito, L'Aquila, Italy). Plasma homocysteine was measured by use of a high-performance liquid chromatography method described elsewhere (20).

Quantification of L-Arginine and Dimethylarginine Concentrations

Concentrations of L-arginine and dimethylarginines in plasma were determined by high-performance liquid chromatography that used precolumn derivatization with *o*-phthalaldehyde, as in a method described elsewhere (2), after extraction of plasma samples on solid-phase extraction cartridges (CBA Varian, Harbor City, CA). The coefficients of variation of this method were 5.2% within-assay and 5.5% between-assay; the detection limit of the assay was 0.1 μmol/L. The plasma concentration of ADMA in normal subjects has a positively skewed distribution with a median value of 0.95 μmol/L, and the 90th percentile is 2.2 μmol/L (10), which we considered to be the upper limit of the normal range.

BP Measurements

BP was estimated by averaging all predialysis arterial pressure recordings during the month before the study (total of 12 measurements; *i.e.*, 3/wk) (21).

Statistical Analyses

Data are reported as mean ± SD or as median and interquartile range, as appropriate. In the cross-sectional study, the relationship between plasma ADMA and IMT was tested by univariate analysis and by multiple-regression analysis. This analysis was based on established risk factors for atherosclerosis in patients on dialysis (age, gender, smoking, diabetes, systolic pressure, cholesterol, calcium phosphate product, albumin, CRP, and homocysteine). Significant independent variables were ordered according to their standardized effect, defined as regression coefficient/standard error of the regression (β).

The relationship between plasma ADMA and IMT changes on follow-up was analyzed separately in patients with initially normal values and in those with abnormal values. The rationale of this analytic approach was that the effect of any putative risk factor on IMT is more likely to be revealed in patients with initially normal vascular structure than in those with overt arterial damage at baseline. Such an approach is supported by the notion that, in hypertensive patients, IMT changes over time are inversely related to initial IMT, which implies that the progression of intimal lesions is more frequently observed in patients with initially normal arterial structure (22). The independent effect of ADMA on the evolution of intimal lesions was studied in a reduced multiple-regression model based on univariate predictors of IMT changes.

Results

Demographic, anthropometric, clinical, biochemical, and echo color Doppler data of the study population are given in Table 1. The plasma ADMA concentration (average 4.22 ± 1.73 μmol/L) exceeded the upper limit of the normal range (cutoff, >2.2 μmol/L) in the majority of patients on dialysis (85/90, *i.e.*, 94%).

ADMA and Carotid Echo Color Doppler Study: Baseline Study

On univariate analysis, plasma ADMA was directly related to IMT either unadjusted or adjusted for the DCCA (Figure 1). On multivariate analysis ADMA, age, systolic BP, and plasma homocysteine resulted to be independent correlates of IMT (Table 2). IMT was significantly related to serum CRP on univariate analysis ($r = 0.23$, $P = 0.03$), but this relationship lost statistical strength on multivariate analysis (see Table 2). Plasma ADMA was largely unrelated to the DCCA either on univariate ($r = 0.06$, $P =$ NS) or on multivariate analysis ($\beta = 0.01$, $P =$ NS).

ADMA and IMT: Follow-Up Study

None of the 90 patients who took part in the study was lost to follow-up. During the follow-up period, 18 patients died and 4 patients underwent renal transplantation; therefore, 68 patients could repeat the echo color Doppler study. Overall, there was a strong inverse relationship between baseline IMT and IMT changes ($r = -0.41$, $P = 0.001$), which implies that worsening of carotid atherosclerosis is more likely to occur in patients with initially normal arterial structure than in those with established disease. In patients with initially normal IMT ($n = 21$), IMT changes were closely related to plasma ADMA ($r = 0.51$, $P = 0.02$, Figure 2A) as well as to CRP ($r = 0.54$, $P = 0.01$, Figure 2B). Furthermore, ADMA and serum CRP were strongly interrelated ($r = 0.64$, $P = 0.002$). IMT changes in this group were largely independent of other risk factors (Table 3). In a multivariate model that included the two significant correlates of IMT changes (ADMA and CRP) and their interaction term, the interaction term ADMA-CRP was the only significant predictor of IMT changes ($\beta = 0.55$, $P = 0.009$), whereas the independent associations between ADMA ($P = 0.38$) and CRP ($P = 0.57$) and IMT changes were largely NS. Plasma ADMA as well as serum CRP were unrelated to changes in DCCA ($r = 0.08$, $P =$ NS and $r = 0.20$, $P =$ NS). In patients with abnormal IMT at baseline (IMT > 0.95 mm), no significant relationship was found between ADMA and

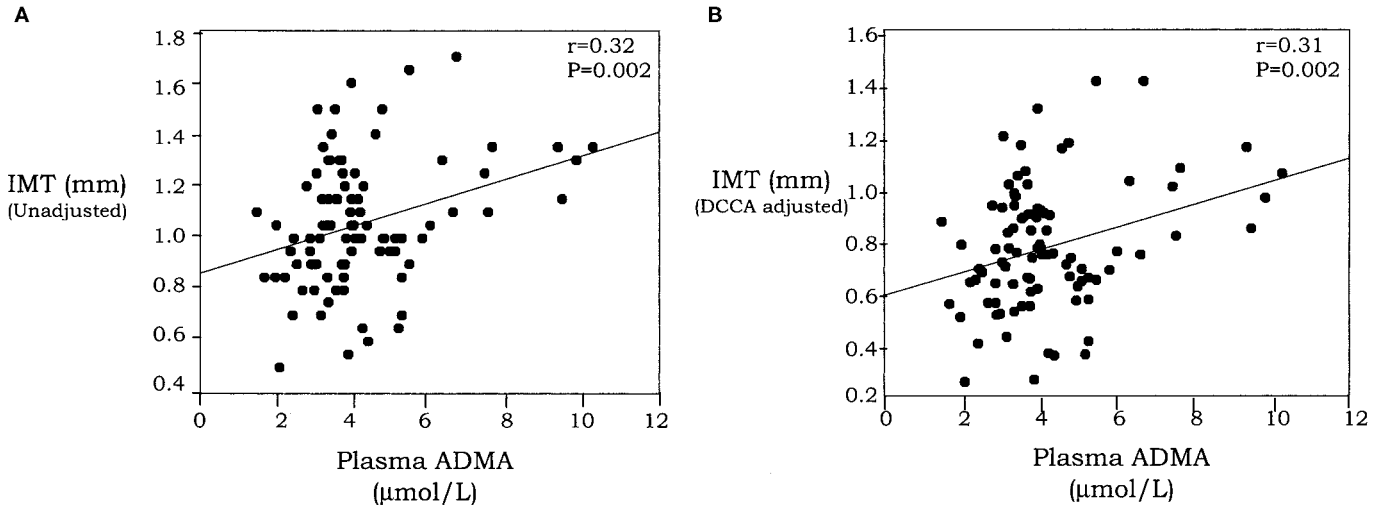

Figure 1. Relationship between plasma asymmetric dimethylarginine (ADMA) and intima-media thickness (IMT) either unadjusted (A) or adjusted for the diameter of the common carotid artery (DCCA; B).

IMT, plaque number, or DCCA changes ($r = -0.004$, 0.17, and -0.16, respectively).

Discussion

Plasma ADMA emerged as an independent correlate of IMT in a large group of patients with ESRD. The interaction between this substance and serum CRP predicted IMT changes at follow-up in patients with normal IMT at baseline. These results lend further support to the hypothesis that ADMA is involved in the high cardiovascular risk of these patients.

Patients enrolled in this study represented a typical sample of the uremic population, and ~11% of them had had major cardiovascular events (11). The mortality rate in these patients is 10%/yr (11), which is close to the mortality rate of patients included in the Italian and European dialysis registries (23). Previous atherosclerotic complications represent a strong predictor of incident cardiovascular events. We have recently shown that IMT in patients on dialysis is a powerful predictor of adverse cardiovascular outcomes (24). Furthermore, it is well known that the severity of carotid atherosclerosis as evaluated by echo color Doppler studies is related to the severity of the atherosclerotic process in other arterial districts, including the coronary tree (25). The fact that, in our survey, the relationship between ADMA and IMT was largely independent of previous cardiovascular complications as well as of traditional and nontraditional risk factors might imply that this association underlies a pathogenetic link. In several previous studies, ADMA levels have been shown to be elevated in chronic renal failure, although absolute values have varied considerably between studies (7–10). This is mainly due to differences in analytic methods applied, because ADMA is protein-bound in plasma (R. H. Böger *et al.* unpublished observation). Some groups used methods that detected unbound rather than total plasma ADMA, which resulted in lower levels than those measured in this study.

In the follow-up study we found that, in patients with initially normal IMT, ADMA, and CRP were related to IMT changes at follow-up and the interaction between these two factors resulted to be the sole independent predictor of progression of intimal lesions. Inflammation is a major factor contributing to atherosclerosis both in the general population and in patients on dialysis (16,26,27). Moreover, inflammatory cytokines may lead to expression of inducible NO synthase (28), which is able to produce NO at high rates and in parallel with superoxide radicals. NO and superoxide can combine to form peroxinitrite (29), a radical with potentially deleterious effects on the vascular wall. Peroxinitrite formation also results in chemical inactivation of NO, which will lead to further

Table 2. Multiple linear regression analysis of intima-media thickness in the survey study[a]

Independent Variables	β	P
Significant predictors		
age	0.41	0.0001
ADMA	0.23	0.01
systolic pressure	0.22	0.04
homocysteine	0.21	0.05
Not significant predictors		
gender	0.18	0.13
albumin	−0.18	0.17
calcium * phosphate	−0.07	0.45
smoking	−0.08	0.49
CRP	0.07	0.50
diabetes	−0.04	0.63
DCCA	−0.04	0.75
cholesterol	−0.02	0.88

[a] Data were expressed as standardized regression coefficients (β) and P values. DCCA, internal diameter of the common carotid artery.

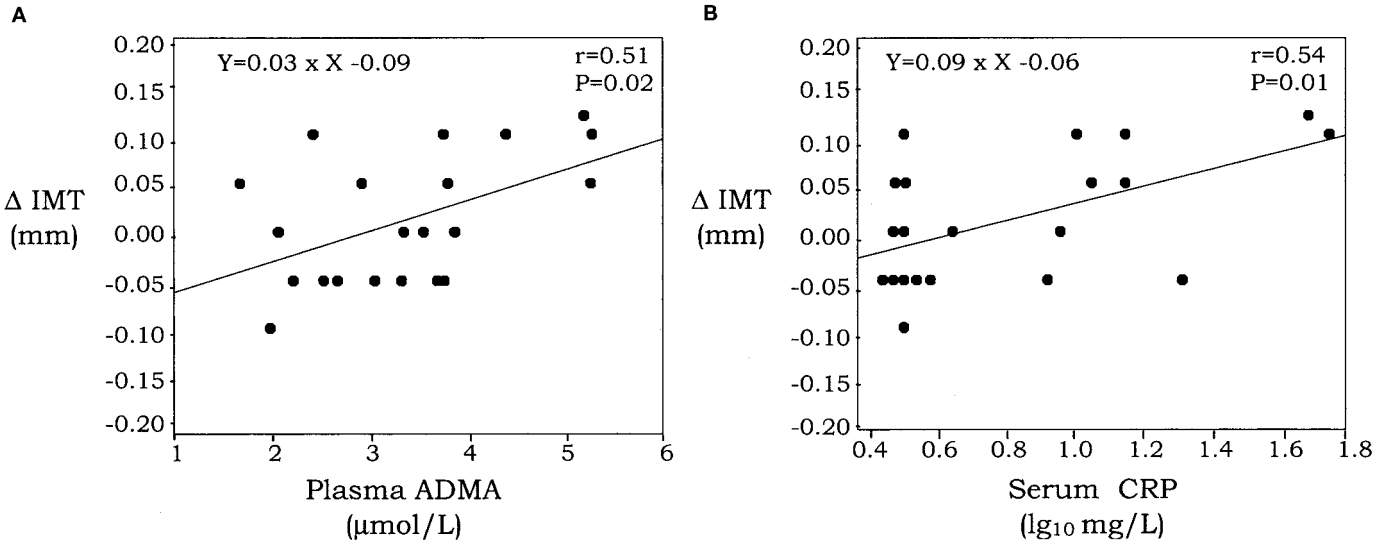

Figure 2. Relationships between baseline plasma ADMA (A) and serum C-reactive protein (CRP; B) and changes in IMT during follow-up.

reduction of the biologic activity of NO beyond inhibition of its formation by ADMA.

At variance of IMT, DCCA was unrelated to ADMA. IMT and other carotid artery lesions, like DCCA, most likely reflect different events occurring in the arterial wall (18) in response to aging and other risk factors and therefore may be linked with different strengths to cardiovascular risk factors. Of note, we have recently shown that IMT, but not DCCA, predicts cardiovascular outcomes in patients on dialysis (24). The reason for elevation of plasma ADMA in patients with uremia has not yet been fully resolved: retention due to impaired renal excretory function may be involved in accumulation of both ADMA and symmetric dimethyl arginine (7,10), but it cannot fully explain increased ADMA levels (30). Metabolism of ADMA by the enzyme dimethylarginine dimethylaminohydrolase may also be impaired in uremia (10,30). We were unable to measure dimethylarginine dimethylaminohydrolase activity in this study because of the unavailability of a sufficiently sensitive method to assess this enzyme's activity in human plasma. Further experimental studies will be needed to assess the contribution of dimethylarginine dimethylaminohydrolase activity to the regulation of ADMA levels in renal and other diseases.

Table 3. Follow-up study: relationship between intima-media thickness (IMT) changes and risk factors in patients with initially normal arterial structure[a]

Risk Factor	Changes in IMT *r* (*P*)
Age	0.15 (0.50)
Gender	0.16 (0.50)
Smoking	0.11 (0.63)
Systolic pressure	0.10 (0.67)
Cholesterol	0.06 (0.79)
Albumin	0.21 (0.35)
Calcium * Phosphate	−0.16 (0.49)
Homocysteine (lg_{10})	0.05 (0.83)
CRP (lg_{10})	**0.54 (0.01)**
ADMA	**0.51 (0.02)**

[a] Data were expressed as Pearson product moment correlation coefficients (*r*) and *P* values. Significant correlation are shown in boldface type.

Although there is a large discrepancy in the literature with regard to the range of ADMA levels in patients with renal failure (7–13), there is substantial agreement that the plasma concentration of ADMA is higher in patients with uremia than in subjects with normal renal function. It remains matter of controversy whether this elevation of ADMA has pathophysiologic significance. At concentrations like those found in our study, ADMA has been shown to inhibit NO synthase activity *in vitro* (5,7). The results of at least three studies are consistent with the hypothesis that endogenous inhibitors of NO synthase are responsible for endothelial dysfunction in patients with uremia (31–33), but no evidence in favor of this hypothesis emerged in a recent study of normotensive patients on dialysis (34). Cardiac and arterial remodelling are strongly linked to endothelial dysfunction in ESRD (6). This study and recent evidence showing that ADMA is a strong predictor of cardiovascular events and of mortality (11) clearly indicate that ADMA is a consistent marker of the severity of atherosclerosis in ESRD. Properly designed prospective intervention studies will establish whether ADMA, CRP, and atherosclerosis are causally linked in patients on dialysis.

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