

## Low dialysance of asymmetric dimethylarginine (ADMA) – in vivo and in vitro evidence of significant protein binding

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### Key words

asymmetric dimethylarginine – dialyzer clearance – nitric oxide – uremic toxin

**Abstract.** **Background:** Increased blood levels of the endogenous nitric oxide synthase inhibitor asymmetric dimethylarginine (ADMA) predict cardiovascular mortality in patients with end-stage renal disease. Despite its low molecular weight, available information on the impact of hemodialysis (HD) on ADMA plasma levels is controversial. **Methods:** We assessed plasma concentrations, dialyzer clearance and total amount of ADMA removed in 30 patients with end-stage renal disease during regular HD. In addition, plasma ADMA levels were assessed in 10 patients with acute renal failure treated with extended HD. **Results:** Regular HD decreased plasma creatinine (from  $774 \pm 42$  to  $312 \pm 17$   $\mu\text{mol/l}$ ) and urea (from  $24.5 \pm 1.5$  to  $8.4 \pm 0.5$   $\text{mmol/l}$ ) concentrations significantly (both  $p < 0.001$ ), whereas plasma ADMA remained unchanged ( $4.35 \pm 0.19$  vs.  $4.76 \pm 0.24$   $\mu\text{mol/l}$ ). ADMA clearance was  $92 \pm 6$   $\text{ml/min}$ , and the total amount removed in the spent dialysate was  $37 \pm 4$   $\mu\text{mol}$ . The clearances of creatinine ( $161 \pm 3$   $\text{ml/min}$ ) and of urea ( $173 \pm 3$   $\text{ml/min}$ ) were significantly higher. Furthermore, even during extended HD, plasma ADMA concentrations did not decrease significantly ( $1.73 \pm 0.22$  vs.  $1.63 \pm 0.18$   $\mu\text{mol/l}$ ). **Conclusion:** In conclusion, dialysance of ADMA is markedly lower than expected from its molecular weight because of significant protein binding of the substance. Since markedly increased ADMA blood concentrations have been linked to cardiovascular complications due to atherosclerosis in patients with ESRD, new strategies should be evaluated to remove this putative uremic toxin.

ture atherosclerosis in patients with end-stage renal disease (ESRD) remains an unresolved problem in the management of this population [Baigent et al. 2000, London and Drüeke 1997]. Since there are abundant experimental data that nitric oxide (NO) counteracts key processes of atherosclerosis, endogenous inhibitors of NO synthesis such as asymmetric dimethylarginine (ADMA) have gained considerable interest over the last decade [Cooke et al. 2000]. Plasma ADMA concentrations are markedly increased in patients with ESRD, and may contribute to the process of atherosclerosis in these patients [Anderstam et al. 1997, Cross et al. 2001, Kielstein et al. 1999, 2001, 2002, MacAllister et al. 1996, Schmidt et al. 1999, 2000, Vallance et al. 1992, Zoccali et al. 2001, 2002]. Indeed, a recent prospective longitudinal study in 225 patients with ESRD revealed that ADMA is a strong and independent predictor of overall mortality and of cardiovascular outcome, ranking second behind age but still above several classical risk factors [Zoccali et al. 2001, 2002].

Since ADMA has a low molecular weight (about 202 Dalton) comparable with that of urea, it should be removed by renal replacement therapy resulting in improvement of organ dysfunction and clinical symptoms. However, results of clinical studies concerning the impact of hemodialysis (HD) on ADMA blood levels are controversial. Although some authors report a significant reduction, others found only small decrease or even no change of plasma ADMA levels with HD [Anderstam et al. 1997, Kielstein et al.

### Introduction

The excessive cardiovascular morbidity and mortality due to complications of pre-

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Table 1. Data of patients with ESRD treated with low-flux and high-flux dialyzers during regular HD. ADMA = asymmetric dimethylarginine.

	Low-flux dialyzer	High-flux dialyzer
Number of patients	10	20
Body weight (kg)	60 ± 5	60 ± 2
Blood flow (ml/min)	251 ± 7	251 ± 3
Treatment time (min)	262 ± 11	256 ± 7
ADMA clearance (ml/min)	79 ± 8	99 ± 7
Total ADMA removed (μmol)	30 ± 3	38 ± 4
Urea clearance (ml/min)	175 ± 6	171 ± 3
Creatinine clearance (ml/min)	163 ± 5	160 ± 4

1999, 2001, 2002, MacAllister et al. 1996, Schmidt et al. 1999, 2000].

In order to clarify this issue, we measured pre- and post-HD plasma concentration of ADMA in 30 patients with ESRD during regular 4-hour HD. In addition, we determined dialyzer clearance for the substance, and quantified the total amount removed by measurement of ADMA concentration in the collected spent dialysate. In addition, we assessed ADMA blood levels during extended HD in 10 patients with acute renal failure (ARF) treated in the intensive care unit.

## Patients and methods

### *Studies on regular HD*

The study was approved by the local Ethics Committee and written informed consent was obtained from all patients. We examined 30 Caucasian patients with ESRD without residual diuresis (15 males, 15 females, age 59 ± 3 years, body mass index 21.7 ± 0.7 kg/m<sup>2</sup>, hemoglobin 11.5 ± 0.2 g/l, serum albumin 36.7 ± 0.9 g/l, and serum cholesterol 5.5 ± 0.2 mmol/l). They had been maintained on HD for a median of 66 months (range 6–298 months). They were treated with HD 3 times weekly and were in a stable condition.

Patients underwent dialysis with biocompatible low-flux (n = 10) dialyzers (F6 S, 1.1 m<sup>2</sup>, Fresenius Medical Care, Bad Homburg, Germany) or high-flux (n = 20) dialyzers (F60 S, 1.3 m<sup>2</sup>, Fresenius Medical Care, Bad Homburg, Germany) using the GENIUS batch dialysis system (Fresenius Medical Care, Bad Homburg, Germany) [Fassbinder

et al. 1998]. The technical details of the system are explained in detail elsewhere [Dhondt et al. 2003]. In brief, sterile bicarbonate dialysate is filled into the 75 l tank and is thereafter circulated in a closed loop circuit. During dialysis, fresh dialysate is taken from the top of the tank while the spent dialysate flows back to the bottom. Thus, complete collection of spent dialysate in the same tank after the dialysis session permits measurement of the total amount removed of any substance [Dhondt et al. 2003]. The average dialysis time during the study was 258 ± 6 min, and mean blood flow was 251 ± 3 ml/min. The mean delivered dialysis dose as indicated by the measured urea reduction rate was 66 ± 1%. Blood samples from the arteriovenous fistula were drawn before the start and at the end of the dialysis, i.e. before the dialyzer and tubing were flushed with saline. Additional blood samples were drawn 10 min after the start of HD pre and post dialyzer in order to calculate the dialyzer clearance from the arteriovenous concentration difference and blood flow. After completion of HD, the spent dialysate in the tank was mixed by air insufflation and representative samples were taken for analysis.

### *Extended HD and ADMA plasma levels*

The study protocol was approved by the local Ethics Committee and written informed consent was obtained from all patients or their legal representatives. We examined 10 Caucasian patients (6 males, 4 females, age 56 ± 4 years, body mass index 31.4 ± 3.4 kg/m<sup>2</sup>), who were treated with extracorporeal renal replacement therapy in the intensive care unit because of oliguric/anuric ARF. They underwent extended HD lasting for 8 hours with biocompatible high-flux dialyzers using the GENIUS batch dialysis system (Fresenius Medical Care, Bad Homburg, Germany) [Dhondt et al. 2003, Fassbinder 1998, Lonnenmann et al. 2000]. The average treatment time was 481 ± 2 min, and average blood flow was 161 ± 2 ml/min. Blood samples for measurement of ADMA plasma concentrations were drawn at regular intervals starting 12 hours before HD, during the HD and 8 hours after the end of HD.

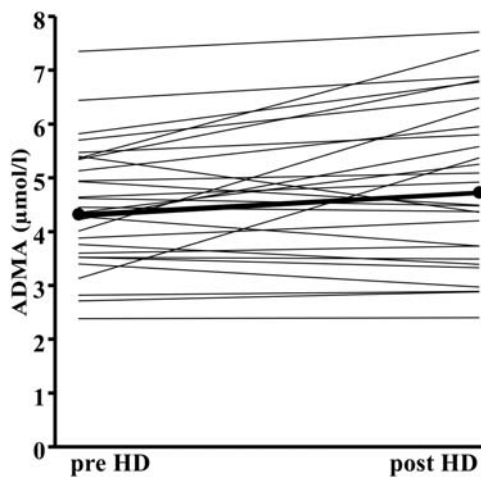


Figure 1a.

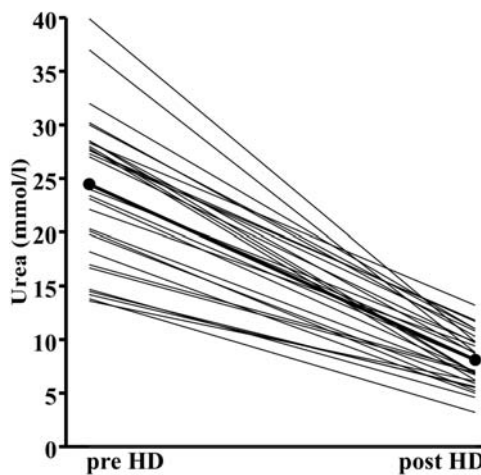


Figure 1b.

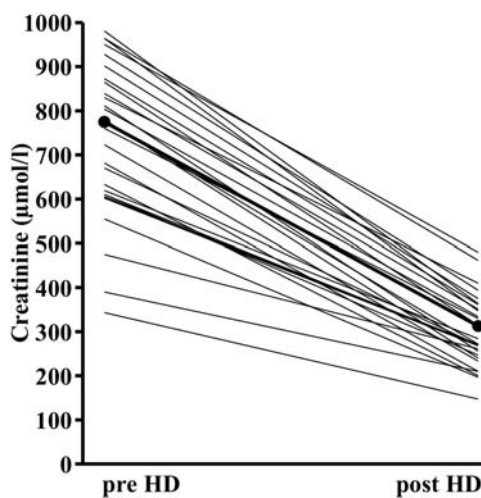


Figure 1c.

Figure 1. Individual pre- and post-hemodialysis plasma concentrations of asymmetric dimethylarginine (ADMA), creatinine and urea in 30 patients with end-stage renal disease treated with regular 4-hour hemodialysis.

### Biochemical analyses

Plasma ADMA levels were determined by high performance liquid chromatography (HPLC) using pre-column derivatization with *o*-phthalaldehyde (OPA) as described previously [Bode-Böger et al. 1996]. Plasma samples and internal standards were extracted on CBA solid-phase extraction cartridges (Varian, Harbor City, CA, USA). The eluates were dried over nitrogen and dissolved in bidistilled water for HPLC analysis. Samples and standards were incubated with the OPA reagent (5.4 mg/ml OPA in borate buffer, pH 8.5 containing 0.4% mercapto-ethanol) before automatic injection into the HPLC. The OPA derivative ADMA was separated on a C6H5 column (Macherey and Nagel, Düren, Germany) with the fluorescence monitor set at an excitation wavelength of 340 nm and an emission wavelength of 455 nm. Samples were eluted from the column with 0.96 citric acid/methanol 2 : 1, pH 6.8 at a flow rate of 1 ml/min. The coefficients of variation of this method are 5.2% within assay and 5.5% between assay, the detection limit of the assay is 0.1  $\mu\text{mol/l}$ . All other laboratory data were obtained from routine laboratory tests using certified assay methods.

For measurement of potential protein binding of ADMA blood samples of 5 ESRD patients were analyzed. Pre- and post-dialyzer samples were taken to explore whether the protein bound portion of ADMA changes during the passage of the blood through the dialyzer. Samples were aliquoted, and one aliquot was deproteinized by ultracentrifugation using millipore CL tubes with a molecular cut off of 10,000 (Millipore, Badford, MA, USA).

### Statistical analysis

In both studies, statistically significant differences between pre- and post-HD data were tested using a paired t-test. Subgroup analysis was performed in ESRD patients treated with low-flux and high-flux dialyzers. Statistical significance was set at  $p < 0.05$ . Data are presented as mean  $\pm$  SEM unless otherwise stated.

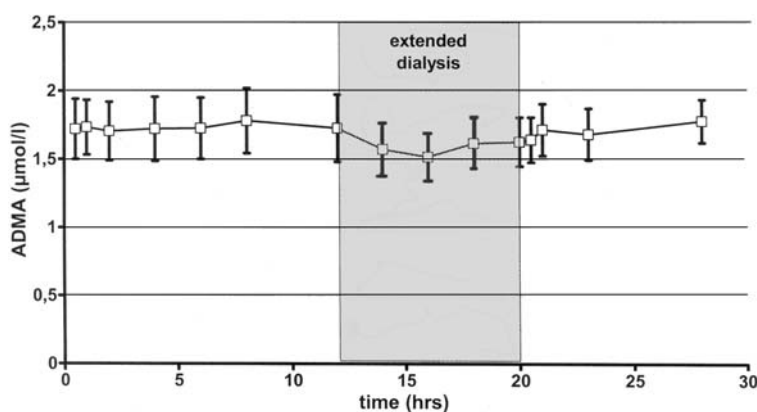


Figure 2. ADMA plasma concentrations in 10 patients with acute renal failure before, during and after treatment with extended HD for 8 hours.

## Results

During 4-hour regular HD, both plasma creatinine concentration decreased significantly from  $774 \pm 42$  to  $312 \pm 17$   $\mu\text{mol/l}$  ( $p < 0.001$ ), and plasma urea levels decreased from  $24.5 \pm 1.5$  to  $8.4 \pm 0.5$   $\text{mmol/l}$  ( $p < 0.001$ ). In contrast, ADMA plasma concentration did not change significantly ( $4.35 \pm 0.19$   $\mu\text{mol/l}$  vs.  $4.76 \pm 0.24$   $\mu\text{mol/l}$ ). Individual data on plasma ADMA, creatinine and urea levels pre- and post-HD are shown in Figure 1a, b, c. As an indicator of hemoconcentration, serum protein concentration increased significantly during HD (from  $67.8 \pm 1.2$   $\text{g/l}$  to  $76.2 \pm 2.1$   $\text{g/l}$ ,  $p < 0.001$ ).

The dialyzer clearances for creatinine and urea were  $161 \pm 3$   $\text{ml/min}$  and  $173 \pm 3$   $\text{ml/min}$ , respectively, whereas that for ADMA was  $92 \pm 6$   $\text{ml/min}$ . A total amount of  $37 \pm 4$   $\mu\text{mol}$  ADMA was recovered in the spent dialysate. No significant differences between low-flux and high-flux dialyzers were observed with respect to ADMA removal (Table 1). Furthermore, the estimated protein binding of ADMA pre- and post-dialyzer was  $90 \pm 4\%$  and  $93 \pm 3\%$ , respectively.

Figure 2 shows mean ADMA plasma concentrations in patients with ARF before, during and after an 8-hour treatment with extended HD. Of note, ADMA plasma levels were remarkably stable and reproducible in these patients over a period of 28 hours (Figure 2). Treatment with extended HD did not have a significant impact on ADMA plasma levels, however.

## Discussion

The striking finding of the present study is that the dialysance and thus removal of ADMA during regular HD is lower than it could be predicted from its molecular weight. This finding was confirmed by measurements of plasma ADMA levels pre and post HD, by calculation of the dialyzer clearance and, of particular importance, by direct measurements of the total amount of ADMA removed during HD. Compared to the dialyzer clearance of urea and creatinine, the clearance for ADMA was markedly lower, although these substances have comparable (low) molecular weights. This was also confirmed by measurements of the total ADMA in the collected dialysate. Such a detailed approach in a large number of patients was made possible by using the GENIUS batch dialysis system, which permits complete assessment of the spent dialysate as has been shown in a recent report [Dhondt et al. 2003]. Furthermore, the results obtained in patients with ESRD on regular HD were confirmed by the finding of almost unchanged ADMA plasma concentrations during prolonged HD treatment in patients with ARF.

Our findings may have important clinical implications. Elevated plasma levels of ADMA in patients with ESRD were first reported by Vallance et al. [1992]. In their study, dimethylarginine levels were elevated about 6-fold compared to healthy controls. Such ADMA blood concentrations are high enough to inhibit NO elaboration by NO synthase in vitro [Faraci et al. 1995, Kurose et al. 1995, Segarra et al. 1999, 2001]. Hence, the well-documented endothelial dysfunction in patients with ESRD could be the consequence of an increased plasma ADMA concentration [Hand et al. 1998, Joannides et al. 1997, Kari et al. 1997]. Several recent studies found markedly elevated plasma ADMA levels not only in patients with ESRD, but also in patients with progressive chronic kidney disease, and even in patients with incipient renal disease and normal renal function [Kielstein et al. 1999, 2001, 2002, Schmidt and Baylis 2000, Xiao et al. 2001]. Although there is overall agreement that plasma ADMA levels in patients with ESRD are markedly increased in relation to those found in healthy controls, the absolute concentration remains

an area of disagreement [Kielstein et al. 1999, 2001, 2002]. However, most recent studies found ADMA plasma concentration between 3 and 6  $\mu\text{mol/l}$ , i.e. consistent with the first report of Vallance et al. [Kielstein et al. 1999, 2001, 2002, Schmidt et al. 1999, 2000, Vallance et al. 1992, Zoccali et al. 2001, 2002]. Irrespective of its absolute concentration, plasma ADMA levels correlate significantly with established risk factors of atherosclerosis not only in renal but also in nonrenal patients [Fard et al. 2000, Kielstein et al. 1999, 2001, 2002, Miyazaki et al. 1999, Yoo and Lee 2001, Zoccali et al. 2001, 2002]. Thus ADMA is thought to be not only a novel biochemical marker of atherosclerosis, but may even be causally involved in the pathogenesis of atherosclerotic disease [Cooke 2000].

The ineffectiveness of HD for removal of ADMA may be in part artefactual, because significant hemoconcentration occurred during HD. These results are consistent with data from our previous study as well as the study of MacAllister et al. [1996] and Kielstein et al. [1999, 2001, 2002]. In both studies, even a slight increase in plasma ADMA concentrations after HD were observed. However, later on, we and others found a decrease of plasma ADMA concentrations [Anderstam et al. 1997, Kielstein et al. 1999, 2001, 2002, MacAllister et al. 1996, Schmidt et al. 1999, 2000]. In none of these studies was the exact time point of blood sampling with respect to the end of the dialysis session reported. Nevertheless, these results are in line with the assumption that the concentration of ADMA in blood is the result of a complex interaction between production, renal excretion and degradation by the enzyme dimethylarginine dimethylaminohydrolase (DDAH). Theoretically, DDAH activity could be altered by HD resulting in variable ADMA degradation. Furthermore, HD-related phenomena such as tissue redistribution and reequilibration of ADMA may complicate this issue even further. In addition, the amount removed could be affected by changes in pH during HD as well. Another reason for the low dialytic clearance of ADMA could be protein binding of this compound. It is of note that our results are in line with a recent study by Achan et al. [2003], who found that in healthy men only 50  $\mu\text{mol}$  of ADMA are eliminated via urinary excretion daily, but 250  $\mu\text{mol}$  are metabolized

by DDAH. It is therefore reasonable to assume that hemodialysis is not more effective with respect to ADMA elimination than the kidney. Whatever the explanation is, ADMA behaves during HD differently from solutes with comparable molecular weight such as creatinine and urea.

In conclusion, dialysance of ADMA is much lower than expected from its molecular weight. Since increased ADMA blood concentrations have been linked to cardiovascular complications due to atherosclerosis in patients with ESRD, new strategies for improved control of circulating levels of this putative uremic toxin have to be assessed.

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