
**UNIVERSITE DE LAUSANNE
FACULTE DE BIOLOGIE ET DE MEDECINE**

Département de Médecine
Division de Physiopathologie Clinique

**Haemodialysis acutely reduces the plasma levels of
ADMA without reversing impaired NO-dependent
vasodilation**

THESE

préparée sous la direction du Professeur Bernard Waeber
et présentée à la Faculté de biologie et de médecine de
l'Université de Lausanne pour l'obtention du grade de

DOCTEUR EN MEDECINE

par

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BME 3543

WG
106
ENG

Lausanne
2009

Bibliothèque Universitaire
de Médecine / BiUM
CHUV-BH08 - Bugnon 46
CH-1011 Lausanne

R005373637

Imprimatur

Vu le rapport présenté par le jury d'examen, composé de

Directeur de thèse *Monsieur le Professeur Bernard Waeber*
Co-Directeur de thèse
Expert *Monsieur le Professeur Renato Panizzon*
Directrice de l'Ecole *Madame le Professeur Stephanie Clarke*
doctorale

la Commission MD de l'Ecole doctorale autorise l'impression de la thèse de

Monsieur Rolf Peter Engelberger

intitulée

*Haemodialysis acutely reduces the plasma levels of ADMA
without reversing impaired NO-dependent vasodilation*

Lausanne, le 27 octobre 2009

*pour Le Doyen
de la Faculté de Biologie et de Médecine*



*Madame le Professeur Stephanie Clarke
Directrice de l'Ecole doctorale*

Remerciements:

Je tiens à remercier très sincèrement toute l'équipe de la Division de Physiopathologie Clinique, particulièrement le Professeur Bernard Waeber et le Professeur François Feihl pour leur engagement exceptionnel et Madame Françoise Bilat pour son aide administrative de valeur, toute l'équipe du Service d'Hémodialyse pour le recrutement des patients dialysés et les prises de sang et Monsieur Hugues Henry pour la mise au point des dosages sanguins.

Un grand merci également à ma famille et mon amie Marie pour leur soutien depuis toujours, qui sans eux, ce travail de thèse n'aurait jamais été possible.



Rolf P. Engelberger

Rapport de synthèse

Introduction: la prévalence de l'insuffisance rénale chronique (IRC) augmente et malgré les traitements de remplacement rénal telle que la transplantation ou la dialyse, la mortalité chez des patients atteints d'une IRC reste très élevée. Les maladies cardiovasculaires sont la cause principale de mortalité chez ces patients, et le risque de décès dû à une complication cardiovasculaire est chez eux accru de 10 à 20 fois par rapport à la population générale. Même si les facteurs de risque cardiovasculaires « traditionnels », principalement l'hypertension artérielle et le diabète sont très prévalents chez les patients avec IRC, ils sont insuffisants pour expliquer l'excès de mortalité cardiovasculaire. D'autres facteurs de risques « non-traditionnels » comme l'accumulation du diméthylarginine asymétrique (ADMA), un inhibiteur endogène de la synthèse d'oxyde d'azote (NO), semblent aussi être importants. Chez les patients avec IRC, des taux élevés d'ADMA sont un puissant facteur prédictif indépendant de la mortalité cardiovasculaire. Il a également été démontré chez des souris que l'ADMA peut être une cause directe de dysfonction endothéliale. Cette dernière joue un rôle primordial dans le développement de l'athérosclérose, cause principale des complications cardiovasculaires. Le but du présent travail est de tester l'hypothèse qu'une réduction du taux d'ADMA après une séance unique d'hémodialyse améliore la dysfonction endothéliale.

Méthodes: la dysfonction endothéliale peut être évaluée dans les microvaisseaux de la peau de façon non invasive par fluxmétrie laser Doppler. La vasodilatation cutanée induite par un échauffement local de 34° à 41°C (hyperémie thermique) est connue pour être dépendante de la production endothéliale de NO et a été utilisée dans plusieurs études cliniques pour évaluer la dysfonction endothéliale. Nous avons recruté 24 patients traités par hémodialyse chronique et également 24 sujets contrôles du même âge et sexe. Chez les patients dialysés, l'hyperémie thermique est mesurée une fois directement avant une séance d'hémodialyse, et une fois directement après une autre séance, toutes deux distantes de 2 à 7 jours. En même temps, les taux plasmatiques d'ADMA sont mesurés par la méthode de spectrométrie de masse en tandem. Chez les sujets contrôle, l'hyperémie thermique est également mesurée à deux reprises, à un intervalle de 2 à 7 jours comme chez les patients dialysés et les taux d'ADMA sont déterminés qu'une seule fois.

Résultats: chez les patients dialysés, les réactions d'hyperémie thermique étaient superposables avant et après dialyse, mais moindre que chez les sujets contrôles. Par contre, les taux d'ADMA étaient plus élevés avant qu'après dialyse. Les taux d'ADMA après dialyse étaient similaires aux taux chez les sujets contrôles.

Conclusion: cette étude montre que la vasodilatation dépendante de la production endothéliale de NO dans la microcirculation cutanée n'est pas influencée par les taux plasmatiques d'ADMA chez les patients dialysés. Ces résultats suggèrent que d'autres mécanismes sont responsables de la dysfonction endothéliale chez ces patients. Ceci met en question le concept que l'accumulation d'ADMA est un facteur causal du risque cardiovasculaire élevé et suggère que l'ADMA est juste un marqueur du milieu très athérogénique causé par l'IRC.

Haemodialysis acutely reduces the plasma levels of ADMA without reversing impaired NO-dependent vasodilation

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A B S T R A C T

End-stage renal disease patients have endothelial dysfunction and high plasma levels of ADMA (asymmetric ω - N^G, N^G -dimethylarginine), an endogenous inhibitor of NOS (NO synthase). The actual link between these abnormalities is controversial. Therefore we investigated whether HD (haemodialysis) has an acute impact on NO-dependent vasodilation and plasma ADMA in these patients. A total of 24 patients undergoing maintenance HD (HD group) and 24 age- and gender-matched healthy controls (Control group) were enrolled. The increase in SkBF (forearm skin blood flow) caused by local heating to 41 °C (SkBF41), known to depend on endothelial NO production, was determined with laser Doppler imaging. SkBF41 was expressed as a percentage of the vasodilatory reserve obtained from the maximal SkBF induced by local heating to 43 °C (independent of NO). In HD patients, SkBF41 was assessed on two successive HD sessions, once immediately before and once immediately after HD. Plasma ADMA was assayed simultaneously with MS/MS (tandem MS). In the Control group, SkBF41 was determined twice, on two different days, and plasma ADMA was assayed once. In HD patients, SkBF41 was identical before ($82.2 \pm 13.1\%$) and after ($82.7 \pm 12.4\%$) HD, but was lower than in controls (day 1, 89.6 ± 6.1 ; day 2, $89.2 \pm 6.9\%$; $P < 0.01$ compared with the HD group). In contrast, plasma ADMA was higher before ($0.98 \pm 0.17 \mu\text{mol/l}$) than after ($0.58 \pm 0.10 \mu\text{mol/l}$; $P < 0.01$) HD. ADMA levels after HD did not differ from those obtained in controls ($0.56 \pm 0.11 \mu\text{mol/l}$). These findings show that HD patients have impaired NO-dependent vasodilation in forearm skin, an abnormality not acutely reversed by HD and not explained by ADMA accumulation.

Key words: asymmetric ω - N^G, N^G -dimethylarginine (ADMA), chronic haemodialysis, endothelium-dependent vasodilation, end-stage renal disease, nitric oxide, skin blood flow.

Abbreviations: ADMA, asymmetric ω - N^G, N^G -dimethylarginine; BMI, body mass index; BP, blood pressure; CKD, chronic kidney disease; CRF, chronic renal failure; d_7 -ADMA, [2,3,3,4,4,5,5- $^2\text{H}_7$]-ADMA; DBP, diastolic BP; DDAH, dimethylarginine dimethylaminohydrolase; ESRD, end-stage renal disease; FMD, flow-mediated vasodilation; HD, haemodialysis; NOS, NO synthase; eNOS, endothelial NOS; PU, perfusion unit; SBP, systolic BP; SkBF, skin blood flow; SkBF_{max}, maximum SkBF; SPE, solid-phase extraction.

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INTRODUCTION

Cardiovascular complications are the most important cause of mortality in ESRD (end-stage renal disease) patients [1]. In this population, one contributing factor to increased cardiovascular risk might be the accumulation of endogenous NOS (NO synthase) inhibitors such as ADMA (asymmetric ω - N^G , N^G -dimethylarginine) [2,3]. This accumulation appears to be due in large part to a down-regulated expression of DDAH (dimethylarginine dimethylaminohydrolase), the enzyme which degrades ADMA [4]. In CKD (chronic kidney disease) patients, plasma ADMA levels are a strong independent predictor of kidney disease progression [5,6] and of all-cause cardiovascular mortality [7]. Furthermore, Matsumoto et al. [8] have demonstrated in a rat model of CRF (chronic renal failure) that ADMA is involved in peritubular capillary loss, tubulointerstitial fibrosis and proteinuria, thereby contributing to the progression of CKD.

ADMA accumulation can be a direct cause of endothelial dysfunction [9], which plays a pivotal role in the development of atherosclerosis [10]. Endothelial dysfunction, manifested by reduced endothelium-dependent vasodilation, exists even with a mild chronic impairment of renal function [11,12], and in uraemia, as in many other pathologies, it has been attributed to reduced bioavailability of endothelium-produced NO [13,14]. Elevated circulating levels of ADMA have been linked to endothelial dysfunction in hypercholesterolaemia [15] and in CKD patients [3,4]. The influence of a single HD (haemodialysis) session on plasma ADMA levels has been described in the literature as highly variable, with a mean decrease after HD ranging from 0 to 84% [16]. The impact of an HD-induced acute fall in plasma ADMA on endothelial NO-dependent vasodilation is not known.

The aim of the present study was to test in ESRD patients the hypothesis that an acute decrease in ADMA levels in plasma after a single HD session may improve endothelial dysfunction in the cutaneous microcirculation.

Part of this work was presented at the XLV ERA-EDTA meeting, Stockholm, Sweden, 10–13 May 2008, and published in abstract form [16a].

MATERIALS AND METHODS

Subjects

A total of 24 patients on maintenance HD for more than 1 month were enrolled (HD group; Table 1). Patients with arteriovenous fistulas on both forearms, dermatological pathologies or treatment with organic nitrates were excluded. All HD patients were anuric except for six of them. The HD technical prescription during the HD investigational sessions is described in Table 2 (Table 2).

Table 1 Demographic and clinical characteristics of the HD patients

Values are means \pm S.D. ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin II type I receptor.

Characteristic	HD group (n = 24)
Age (years)	67.9 \pm 13.6
Gender (male/female) (n)	18/6
BMI (kg/m ²)	25.6 \pm 4.5
Months on HD	41.6 \pm 68.4
Underlying renal disease (n)	
Diabetic nephropathy	9
Hypertensive nephrangiosclerosis	17
Chronic glomerulonephritis	2
Cyclosporine induced nephropathy	2
IgA nephropathy	1
Other/unknown	6
Co-morbidities (n)	
Diabetes	9
Coronary heart disease	8
Hypercholesterolaemia	12
Polyneuropathy	7
Smokers	8
Medication (n)	
β -Adrenergic blockers	8
Calcium-channel blockers	8
ACEIs	7
ARBs	11
Antiplatelet therapy	11
Diuretics	8
Statins	13
Recombinant erythropoietin	19

The dialysate temperature was maintained constant at 37°C. All patients had high-flux filters.

A total of 24 healthy subjects who were matched to the HD group for age and gender were recruited [Control group; age, 68.8 \pm 11.7 years; 17 male and seven female; BMI (body mass index), 24.7 \pm 2.3 kg/m²]. The control subjects were all non-smokers, did not have cardiovascular or kidney disease, diabetes or dermatological disease, and were not taking any medication.

All participants in the present study were Caucasians, as skin colour influences laser Doppler flowmetry.

The study was approved by the ethics committee of the University of Lausanne and conformed to the principles outlined in the Declaration of Helsinki. All participants gave written informed consent.

Assessment of skin endothelial function

The measurement of microvascular SkBF (skin blood flow) with laser Doppler flowmetry can be used for the non-invasive assessment of endothelial function in the human cutaneous microcirculation [17]. Local heating

Table 2 Technical HD prescription

Values are means \pm S.D. $spKt/V_{urea}$, single pool Kt/V_{urea} , according to the second-generation Daudirgas formula [67]. Haemodiafiltration was always online.

Prescription	
Duration of HD (min)	214.8 \pm 23.3
Filter type	
Polysulfone	13 out of 24 patients
Polyamide	11 out of 24 patients
HD	19 out of 24 patients
Haemodiafiltration	5 out of 24 patients
Substitution volume (ml/min)	89.0 \pm 5.7
Blood flow rate (ml/min)	436.3 \pm 59.8
Dialysate flow rate (ml/min)	490.4 \pm 26.9
Ultrafiltration volume (kg)	2.5 \pm 0.8
$spKt/V_{urea}$	1.4 \pm 0.2

of the skin to temperatures below 42 °C typically induces a biphasic increase in SkBF with an initial peak and a secondary sustained plateau after 20–30 min (thermal hyperaemia). This plateau response is known to be primarily mediated by the local generation of NO [18], with recent findings supporting the production by eNOS (endothelial NOS), rather than nNOS (neuronal NOS) [19]. Thermal hyperaemia has been used in clinical studies to investigate endothelium-dependent NO release [20]. Its decrease has been shown to be of prognostic value for cardiovascular mortality in ESRD patients [21].

Measurement of SkBF

Laser Doppler flowmetry was carried out with a laser Doppler imager (LDI; Moor Instruments), as described previously [22,23]. This device allows the measurement of dermal microvascular blood flow in a region of interest with no skin contact. According to the principles of laser Doppler flowmetry, SkBF is expressed in PUs (perfusion units).

Local thermal hyperaemia

The change in SkBF induced by local warming of the skin was recorded by means of temperature-controlled water-filled chambers as described previously [23,24]. Local temperature was set at 34 °C until SkBF was stable, then was raised to 41 °C in 60 s and maintained at this level for 28 min. Subsequently, the temperature was increased to 43 °C for another 15 min to induce SkBF_{max} (maximum SkBF) [25].

Analytical methods

Plasma levels of ADMA

ADMA determination in plasma was performed by electrospray MS/MS (tandem MS). To 60 μ l of plasma, 30 μ l of d₇-ADMA ([2,3,3,4,4,5,5-²H₇]-ADMA) at 1 μ mol/l (Cambridge Isotope Laboratories), 106 μ l of water and

4 μ l of formic acid were added. The analytes were purified by μ -SPE (micro-solid-phase extraction) using the Oasis MCX μ Elution Plate (Waters). The procedure comprised of what is typical for SPE: conditioning of the μ -SPE wells by methanol, application of the diluted samples, wash steps with 2 % (v/v) formic acid and methanol, and finally elution with 1.25 % (v/v) NH₄OH in water.

Chromatographic analysis was performed using a Rheos CPS-LC (Flux Instruments) and a PAL SYSTEM autosampler (CTC Analysis). Separation of the analytes was achieved at 30 °C using an Atlantis HILIC silica 2.1 \times 50 mm column (Waters). The mobile phases used for the chromatographic separation were composed of 20 mmol/l ammonium formate, 0.4 % (v/v) formic acid in water and acetonitrile. The column effluent was monitored using a Triple Quadrupole TSQ Quantum Discovery (Thermo Scientific). The instrument was equipped with an electrospray interface and was controlled by the Xcalibur software (Thermo Scientific). The samples were analysed in the positive-ionization mode operating in a cone voltage of 4 kV. The tandem mass spectrometer was programmed using the SRM (selected reaction monitoring) mode to allow the [MH⁺] ions of ADMA at *m/z* 203 and that of the internal standard d₇-ADMA at *m/z* 210 to pass through the first quadrupole (Q1) and into the collision cell (Q2). The daughter ions for ADMA and d₇-ADMA are of *m/z* 46. Calibration curves were computed using the ratio of the peak area of the analytes and internal standard using a weighted (1/*x*²) least-squares linear regression analysis.

We did not assay the symmetric form of dimethyl-L-arginine [SDMA (symmetric ω -N^G,N^G-dimethylarginine)], which is devoid of inhibitory effect on NOS activity [3].

Plasma levels of L-arginine

For practical reasons, L-arginine levels in plasma were measured using a protocol in our laboratory based on HPLC. The analysis was performed using an Agilent 1100 HPLC system. A reverse-phase was used for chromatographic separation [Zorbax Eclipse C₁₈ column AAA (4.6 \times 150 mm; 3.5 μ mol/l); Agilent Technologies]. Detection was done by fluorescence measurements at 340 nm (excitation) and 450 nm (emission). ChemStation Rev B 03-01 software (Agilent Technologies) was used for data acquisition and analysis.

An internal standard (50 μ l of 1250 μ mol/l δ -glucosaminic acid in 160 g/l 5-sulfosalicylic acid) was added to 200 μ l of plasma. The samples were mixed thoroughly and incubated on ice for 1 h. The tubes were centrifuged at 20800 g. Then, 30 μ l of 200 mmol/l Na₂HPO₄ (pH 7.8) was added to 120 μ l of supernatant. The samples were transferred into HPLC vials for injections.

Automatic pre-column derivatization with OPA-3MPA was performed at room temperature (21–24 °C)

using an autosampler program in accordance with the manufacturer's instructions (Agilent Technologies). The mobile phase A was 40 mmol/l NaH_2PO_4 (pH 7.8), and mobile phase B was acetonitrile/methanol/water (9:9:2, by vol.). The separation was obtained at a flow rate of 1 ml/min with a gradient program that allowed for 2 min of 0% B, followed by a 26 min step that raised mobile phase B to 20%. Then, washing at 100% B for 10 min and the equilibration steps at 0% B for 10 min were performed for a total run of 48 min.

Experimental protocol

HD group

Each subject was examined on two separate visits 2 or 3 days apart. Caffeine-containing beverages were avoided on the day of the experiments, and smokers were not allowed to smoke for at least 8 h before examination. The visits were on two successive HD sessions. Thermal hyperaemia was recorded, and blood was drawn for the determination of plasma ADMA and L-arginine at the same visit, once in the hour before and once immediately after HD, in a randomized order. The blood sample after the HD session was collected after a short equilibration period, as usually determined in order to measure Kt/V_{urea} [dialysis quality; where K is dialyser clearance for urea, t is time, and V_{urea} is the urea distribution volume (i.e. total body water)] in a standardized fashion. This was as follows: dialysate flow stopped, ultrafiltration stopped, blood flow reduced to 100 ml/min for 3 min, and blood taken from the arterial line. Twelve patients attending the morning HD session were thus examined in the morning period, and the 12 patients attending the afternoon HD session were assessed in the afternoon. Investigations were not performed before and after the same HD session for the following reasons: (i) we wanted to record thermal hyperaemia on exactly the same skin site on both occasions in order to avoid potential confounding by spatial variation of the vasodilatory response [22], and (ii) we have recently published results indicating that thermal hyperaemia recorded on a particular site can be diminished by previous heating of this site carried out a few hours before [24]. Measurements were performed in the forearm contralateral to the arteriovenous vascular access. After an acclimatization period of 10 min, the subjects were examined in the supine position with the forearm under investigation supported by a vacuum splint. The exact location of the site on the examined forearm was marked on a transparent acetate film, together with the anatomical outline of the forearm, in order to exactly locate the same site for the SkBF measurements performed during the second visit [22].

BP (blood pressure) and heart rate were measured before and after the recording of thermal hyperaemia on the contralateral arm using an automated electronic device (Datascop Accutorr 1A; MS Cardio-Medical).

Skin temperature was also systematically measured using a cutaneous probe (G. Métraux). The investigations were carried out in a quiet room with air conditioning. Ambient temperature was systematically measured and ranged from 23–26°C.

Control group

Each participant in this group also had two separate measurement sessions at the same time of day as in the HD group (sessions 'before' and 'after'), also on two different days. The experimental protocol was identical with that followed in the HD group, except for the absence of HD and for the fact that plasma ADMA was determined only once.

Data analysis

Values are presented as means \pm S.D., or medians (range) in the case of asymmetrical distributions. To take into account the biphasic SkBF response to local heating [18,23], local thermal hyperaemia was summarized as: (i) baseline (average SkBF of the first two measurements); (ii) initial peak (maximal SkBF in the first 6 min after the beginning of heating); (iii) nadir (minimal SkBF after the initial peak); (iv) plateau (average SkBF of the last 5 min at 41°C); and (v) SkBF_{max} (average SkBF of the last 5 min at 43°C). SkBF_{max} is expressed in PUs, whereas all of the other SkBF measurements are expressed as a percentage of SkBF_{max} for normalization purposes [17]. Contrary to plateau SkBF, SkBF_{max} is independent of endothelial NO, as are the initial peak and the nadir [18,19].

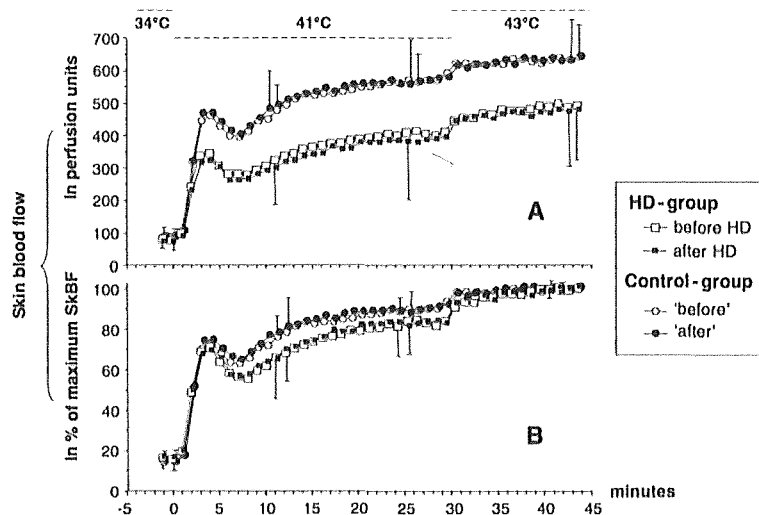
Statistical analysis of the SkBF data was carried out with univariate repeated-measures ANOVA. When the F value was significant, pairwise comparisons were carried out with modified Student's t tests (i.e. Fisher-protected least-significant difference). The α level of all tests was set at 0.05. All computations were performed with JMP software version 5.0.2 (SAS Institute).

RESULTS

Vital parameters and skin temperature values are summarized in Table 3. SBP and DBP (systolic and diastolic BPs respectively) were significantly higher in HD patients than in control subjects ($P < 0.01$). Compared with the Control group, mean BP in the HD group was significantly higher before ($P < 0.01$), but similar after, the HD session. HD did not affect heart rate, which was significantly higher in HD patients than in controls ($P < 0.01$). Skin temperature at the forearm level was slightly lower in HD patients than in controls ($P < 0.05$) and was not influenced by HD. This difference may be explained, at least in part, by a somewhat lower ambient temperature in the examination room used with the HD patients, which for practical reasons was located within

Table 3 Vital parameters and response of SkBF to local heating (41 °C)Values are means \pm S.D. ** $P < 0.01$ compared with after HD. $^{\circ}P < 0.05$ and $^{\circ\circ}P < 0.01$ compared with the Control group.

Parameter	HD group		Control group	
	Before HD	After HD	'Before'	'After'
Vital parameter				
SBP (mmHg)	146 \pm 23** $^{\circ\circ}$	125 \pm 20	124 \pm 20	125 \pm 18
DBP (mmHg)	76 \pm 11** $^{\circ\circ}$	66 \pm 14 $^{\circ}$	70 \pm 14	71 \pm 11
Mean BP (mmHg)	99 \pm 13** $^{\circ\circ}$	86 \pm 13	88 \pm 13	89 \pm 14
Heart rate (beats/min)	75 \pm 13 $^{\circ\circ}$	74 \pm 12 $^{\circ\circ}$	63 \pm 8	61 \pm 7
Weight (kg)	75.8 \pm 17** $^{\circ\circ}$	73.6 \pm 16.4	73.2 \pm 10.8	73.2 \pm 10.8
Skin temperature (°C)	32.5 \pm 0.8 $^{\circ}$	32.6 \pm 1.0 $^{\circ}$	33.2 \pm 1.1	33.3 \pm 1.1
Ambient temperature (°C)	24.2 \pm 0.4 $^{\circ}$	24.6 \pm 0.6 $^{\circ}$	24.8 \pm 0.7	25.0 \pm 0.8
SkBF response				
Baseline (% of SkBF _{max})	16.1 \pm 4.9	16.7 \pm 6.1	14.4 \pm 5.5	14.7 \pm 6.0
Initial peak (% of SkBF _{max})	73.4 \pm 13.3	72.4 \pm 20.4	74.2 \pm 8.6	78.6 \pm 10.6
Nadir (% of SkBF _{max})	51.5 \pm 15.5	50.5 \pm 17.9	59.0 \pm 9.9	59.0 \pm 14.2
Plateau (% of SkBF _{max})	82.2 \pm 13.1 $^{\circ\circ}$	82.7 \pm 12.4 $^{\circ\circ}$	89.6 \pm 6.1	89.2 \pm 6.9
SkBF _{max} (PUs)	491 \pm 163 $^{\circ\circ}$	475 \pm 174 $^{\circ\circ}$	632 \pm 92	635 \pm 129

**Figure 1** Response of SkBF to local heating in patients on chronic HD for ESRD and in control subjects

(A) SkBF is expressed in PUs; (B) SkBF is expressed as a percentage of the maximum SkBF at 43 °C. Patients on HD and control subjects were matched for age and gender. Before HD, the hour that preceded one HD session; after HD, the hour that followed another HD session; 'before' and 'after', two measurements made 2 or 3 days apart in the same control subject at the same time of day as in the patients. The two HD sessions (or in controls the two measurement sessions) were 2 or 3 days apart. Values are means \pm S.D. of 24 subjects per group.

the HD ward, whereas the control subjects were studied in the physiology laboratory.

As shown in Figure 1 and Table 3, SkBF_{max} [i.e. elicited by heating at 43 °C (NO-independent) and expressed in PUs] was significantly lower in the HD group than in the Control group, and completely unaffected by HD. When expressed as a percentage of SkBF_{max}, the plateau SkBF [i.e. after 30 min of heating at 41 °C (NO-dependent)] was significantly lower in HD patients than in controls, whereas the initial peak and the nadir responses (both independent of endothelial NO production [18,19]) did

not differ between the groups. None of the variables derived from thermal hyperaemia was affected by HD. In the Control group, the first and second thermal hyperaemia were identical in every respect.

Measurements in the morning did not differ from those in the afternoon in both HD patients and the Control group (results not shown). There was no correlation between the temperature of unheated skin (measured in the vicinity of the thermally probed area) and any of the parameters derived from thermal hyperaemia. This lack of correlation makes it very unlikely that the slightly lower

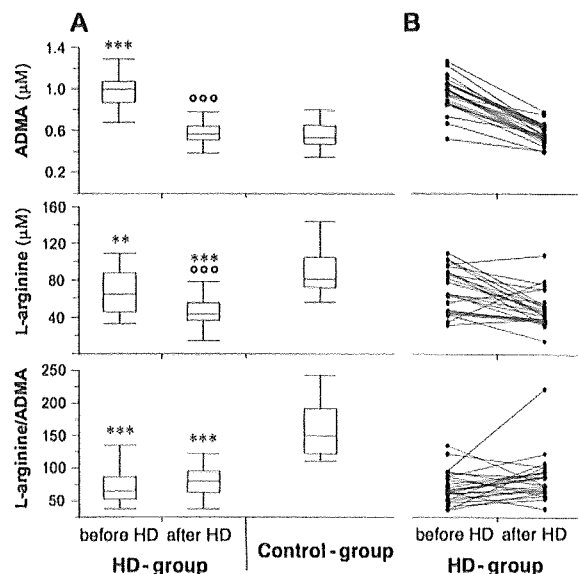


Figure 2 Effects of HD on plasma levels of ADMA and L-arginine

In patients on HD, plasma ADMA and L-arginine levels were assessed in the hour that preceded one HD session (before HD) and immediately after another HD session (after HD). Plasma ADMA was obtained only once in the Control group. (A) Boxes indicate the 25th, 50th and 75th percentile, and whiskers indicate the range. (B) Individual changes before and after HD. ** $P < 0.01$ and *** $P < 0.001$ compared with the Control group; $\circ\circ\circ P < 0.001$ compared with 'before HD'.

unheated skin temperature of the patients could account for the differences between the groups shown in Figure 1 and Table 3.

Blood was drawn for the determination of plasma ADMA and L-arginine levels simultaneously with SkBF measurements on both sessions in the HD group, and on only one session in the Control group (Figure 2). In the HD group, ADMA and L-arginine levels were higher before HD (0.98 ± 0.17 and $70 \pm 24 \mu\text{mol/l}$ respectively) than after HD (0.58 ± 0.10 and $49 \pm 19 \mu\text{mol/l}$ respectively; $P < 0.001$ for ADMA and $P = 0.005$ for L-arginine). Before HD, ADMA levels were also significantly higher and L-arginine levels significantly lower than in the Control group (0.56 ± 0.11 and $88 \pm 23 \mu\text{mol/l}$; $P < 0.001$ for both analytes). After HD, ADMA levels of the patients did not differ from the values of control subjects, whereas L-arginine was considerably lower ($49 \pm 19 \mu\text{mol/l}$; $P < 0.001$). The median relative decreases in plasma ADMA and L-arginine levels induced by HD were -40 and -29% respectively, of the pre-dialysis value, with a substantial variability between patients (range for ADMA, -20 to -52% ; range for L-arginine, -66 to $+117\%$) (Figure 2B).

The plasma concentration L-arginine/ADMA ratio has been advocated as surrogate for NO production capacity [15]. In patients, this ratio was considerably lower than in

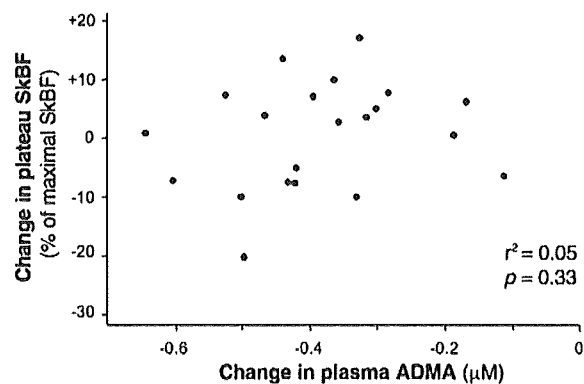


Figure 3 Correlation between changes in plasma levels of ADMA induced by HD and the corresponding changes in the plateau SkBF response to local heating (41°C)

Changes in SkBF are expressed as a percentage of SkBF_{max} elicited by subsequent heating to 43°C (see Figure 1).

control subjects [before HD, 66 (38–136) compared with 150 (1120–242) in the Control group; $P < 0.001$] and there was a small, but non-significant, increase in this ratio after HD [80 (38–222); $P = 0.06$] (Figure 2).

There was no correlation between the changes observed between the two HD sessions in the NO-dependent SkBF plateau and the corresponding changes in plasma ADMA ($r^2 = 0.05$, $P = 0.33$; Figure 3), plasma L-arginine ($r^2 = 0.00$, $P = 0.95$) and L-arginine/ADMA ratio ($r^2 = 0.02$, $P = 0.56$).

DISCUSSION

In the present study, we have found that HD markedly and transiently decreased the plasma levels of both L-arginine and ADMA, with only a small non-significant increase in the L-arginine/ADMA ratio. Concomitantly, there was no acute influence of HD on an NO-dependent vasodilatory response in the skin microcirculation. These results raise the possibility that, in ESRD patients, removal of endogenous NOS inhibitors by HD is not efficient in improving endothelial function due to the concomitant changes induced in the availability of L-arginine. Alternatively, mechanisms other than or in addition to inhibition of eNOS by accumulated ADMA may be responsible for endothelial dysfunction in ESRD.

Plasma L-arginine and ADMA levels

Pre-dialysis values

In the HD group before dialysis, plasma L-arginine was 30% lower and plasma ADMA was 40% higher in comparison with values from the Control group, which was matched for age and gender. Although not reported constantly [26–28], an abnormally low level of circulating L-arginine has been a frequent finding in previous studies

of ESRD patients [29–33], possibly related to deficient nutritional status [31], diminished L-arginine synthesis by the kidney, chronic loss of this amino acid in the dialysate [34], or stimulated transport into cells via the y^+ cationic amino-acid transporter [33].

Retention of ADMA has been uniformly found in ESRD patients, although the relative increase in the plasma level of this arginine analogue has varied between studies from 1.3 to 10 times the normal value (reviewed in [16]). Our pre-dialysis and control ADMA results are in agreement with those obtained either with HPLC by Fleck et al. [29], Wahbi et al. [35] and Schiel et al. [36] or with LC-MS (as in the present study) by Martens-Lobenhoffer et al. [37].

Acute impact of HD

In ESRD patients, the short-term effect of a single HD session on the plasma levels of L-arginine has been extremely variable between studies, with some finding no change [27,28,32,38] while others reported decreases ranging from 10–70% of pre-dialysis values [26,39–41]. The present study clearly belongs to the second category (Figure 2), although the results differed considerably between patients, possibly because of a variable redistribution of L-arginine between intracellular and extracellular pools (keeping in mind that the former is by far the larger) [42].

A recent systematic review identified 19 clinical studies where the acute impact of HD on ADMA plasma levels was documented in ESRD patients [16]. Findings were highly variable between studies, with a mean percentage reduction ranging from 0–84%. [16]. In the present study, HD appeared efficient at decreasing ADMA plasma levels (a mean decrease of 40%), although there was substantial variability between patients in that respect. Low ADMA clearance during HD compared with other substances of similar chemical structure might be explained by significant protein binding [43]. The influence of oxidative stress, pro-inflammatory cytokines and pH changes on catabolism of ADMA as well as on its uptake and release by erythrocytes [44] or other cells [45] may also be involved.

Thermal hyperaemia as a non-invasive means to evaluate NO-dependent vasodilation in CKD

Two clinical studies have found that microvascular endothelial function, as assessed with local thermal hyperaemia, was depressed in ESRD patients [21,46], and the present study confirms these results (Table 3). Furthermore, a prospective study of CRF patients showed that impaired skin thermal hyperaemia was predictive of cardiovascular mortality after 2 years, independently of other conventional risk factors [21]. Collectively, these findings support the validity of a

diminished plateau of thermal hyperaemia as an indicator of endothelial dysfunction in the setting of CKD.

In our present study, the interpretation of differences in plateau $SkBF$ at 41°C between patients and controls is complicated by the unequal vasodilatory reserve of the skin microcirculation in the two groups, as shown by the markedly different $SkBF_{max}$ (Figure 1 and Table 3). In contrast with the submaximal $SkBF$ plateau, $SkBF_{max}$ is insensitive to NOS inhibitors [18] and is thought to depend directly on the local density of microvessels (i.e. the vasodilatory reserve) [47]. In view of the well-established association between hypertension and rarefaction of skin capillaries [48] and considering the markedly higher BP in the HD group compared with the Control group (Table 3), it appears likely that the lower $SkBF_{max}$ measured in patients on HD was due, at least in part, to the hypertension-related reduction in skin capillary density. Another possible explanation relates to the well-known angiogenic effect of NO [49]. In the mouse, chronic administration of a NOS inhibitor induced capillary rarefaction, at least in the kidney medulla [50]. Thus microvascular rarefaction in the HD group might also have been promoted by chronic NO deficiency, which is characteristic of CKD and can be due, in part, to ADMA accumulation [34].

In view of these considerations, information on functional (as opposed to structural) mechanisms which potentially participate in the observed difference in thermal hyperaemia between the two study groups can only be extracted by comparing the vasodilatory responses normalized to $SkBF_{max}$, as we have done in the present study. The normalized NO-dependent [18] plateau $SkBF$ at 41°C—was lower in the HD group, but the normalized NO-independent [18] early peak was not (Table 3). These observations support a specific impairment of NO-mediated vasodilation in the skin microcirculation of renal failure patients.

Impact of HD on thermal hyperaemia

The HD prescription included the withdrawal of an amount of fluid sufficient to cause significant hypovolaemia (i.e. a mean ultrafiltration volume of 2.5 litres; Table 2), thus explaining the fall in BP that occurred in the course of the session (Table 3). This in turn may have triggered a baroreflex that could have had an impact upon skin vasoreactivity.

Interestingly, despite an important BP decrease during HD, basal $SkBF$ as well as $SkBF_{max}$ were not affected (Table 3). This suggests stable haemodynamic conditions in the cutaneous microcirculation even with important systemic haemodynamic modifications, making this method an interesting tool for the assessment of microvascular function in various conditions.

The actual impact of HD on endothelial function remains controversial. We did not find any difference in the thermal hyperaemic response before and after HD.

Therefore, at least in the dermal microcirculation, HD does not appear to negatively or positively influence NO-dependent vasodilation in the short term, although its long-term impact in that respect cannot be inferred from our present findings. Our present results are consistent with some, but not all, previous findings made in patients on maintenance HD. The HD session had no effect on FMD (flow-mediated vasodilation) of the brachial artery [51], endothelium-dependent vascular reactivity assessed by applanation tonometry [28] or the response of the skin microcirculation to iontophoretic application of acetylcholine [52], an endothelium-dependent vasodilator. However, some investigators have found that endothelium-dependent vasodilation was acutely reduced after HD, possibly because of the induced oxidative stress [53,54], the amount of which appears to depend on the type of dialysis filter used [55]. Improvement in endothelial function immediately after the HD session has also been reported [26,56,57]; however, we emphasize that, with one exception [52], all of these studies did not specifically assess endothelium-dependent vasodilation in the microcirculation.

ADMA and NO-dependent vasodilation in CKD

In the present study, we observed no correlation between plasma ADMA levels and endothelial NO-dependent vasodilation in the cutaneous microcirculation (Figure 3). More importantly, HD acutely reduced the plasma concentration of ADMA (Figure 2), but did not have any impact on NO-dependent vasodilation (Figure 1).

Our results are consistent with the findings of Chan et al. [58], who observed a complete dissociation between the changes in FMD and the changes in plasma ADMA levels which followed an increase in the dose of dialysis (i.e. a switch from conventional to nocturnal HD). Another study in patients on peritoneal dialysis reported that ADMA levels were related to basal, but not to acetylcholine-stimulated, NO bioactivity in the forearm circulation [59]. Cross et al. [56] did not find any correlation before and after an HD session between plasma ADMA or homocysteine concentrations and FMD and they concluded that the impaired conduit artery endothelial function in ESRD was due to the total burden of circulation inhibitors rather than any single molecular species. On the other hand, Yilmaz et al. [60] found a significant negative correlation between FMD and ADMA levels before and 4 weeks after kidney transplantation, suggesting that increased ADMA levels might be one of the contributors to endothelial dysfunction in ESRD patients. However, their study population was much younger (mean age, 27.9 ± 9.1 years) than those in the studies by Chan et al. [58] (41 ± 2 years), Cross et al. [56] (43 ± 3.5 years) and ours (the present study; 68 ± 14 years), and the dialysis time of these patients

before transplantation was not reported. In CRF, it is conceivable that the contribution of ADMA accumulation to impaired NO-dependent vasodilation varies with patient age and/or disease duration in this setting.

The dissociation between changes in ADMA levels and changes in NO-dependent vasodilation observed in two studies as well as in our present study could be due to a concomitant reduction in NOS substrate availability, as possibly reflected by the acute decrease in plasma levels of L-arginine caused by HD (Figure 2). However, this is not supported by the findings of Cross et al. [56], who examined ESRD patients immediately before an HD session and were unable in these conditions to augment the NO-dependent vasodilation of the brachial artery (FMD) or of the forearm microvasculature (intra-arterial infusion of acetylcholine) by the systemic or intra-arterial infusion of L-arginine [61]. Another explanation might reside in the capacity of endothelial cells to concentrate ADMA. Cellular studies on bovine aortic endothelial cells have shown that intracellular ADMA levels could be 5–10 times higher than outside the cell [45]. There is a continuous low-level production of ADMA from the proteolysis of methylated arginine residues on various proteins [3], but its concentration is kept below a threshold concentration for inhibition of NOS by the activity of DDAH, the enzyme which degrades ADMA [4]. However under specific conditions, such as the addition of ADMA to the medium of cultured endothelial cells [45], administration of exogenous ADMA to experimental animals or humans [62] and DDAH deficiency in mice [9], intracellular ADMA may reach levels sufficient to inhibit NOS function and endothelium-dependent vasodilation. To date, it is not known how the intracellular environment tracks the extracellular concentration of ADMA when the latter varies rapidly. Nor is it clear whether high extracellular ADMA has biological effects in its own right or is merely a marker of high intracellular levels [3]. Alternatively, our results could mean that inhibition of NOS by accumulated ADMA is not the main culprit for reduced NO bioactivity in ESRD patients. Other potential mechanisms include scavenging of NO by ROS (reactive oxygen species), reduced expression of eNOS [4], possibly mediated by the accumulation of advanced glycation end-products [63], deficient supply of L-arginine or inhibition of L-arginine transport into cells [34].

Study limitations

One limitation of the present study is that the potential vascular impact of HD was not tested in isovolaemic conditions, as shown by the substantial ultrafiltration volume withdrawn from patients (Table 2). The resulting decrease in BP (Table 3) may have triggered a baroreflex-mediated sympatho-adrenergic activation, with the resulting vasoconstriction then masking any

positive influence of reduced ADMA levels on thermal hyperaemia. Although not supported by the absence of any effect of HD on basal SkBF (Table 3), this interpretation of our findings cannot be ruled out. In practical terms, however, our results would still imply a limited or non-existent overall impact of acutely reduced ADMA levels on NO-dependent skin vasodilation, since HD is not usually carried out in isovolaemic conditions.

Temperature of the dialysate was kept at 37°C. In such conditions, it is possible that HD caused an increase in core temperature [64], thus adding a complicating influence on the regulation of SkBF [65]. However, if there were core temperature changes in our present study, they appear to have had a minimal influence on the cutaneous microcirculation, since skin temperature as well as basal SkBF were unaffected by HD (Table 3).

Another possible limitation is the fact that microvascular reactivity as well as blood parameters were not determined before and after the same HD session, for the specific reasons indicated in the Materials and methods section. However, this feature of our study design has limited potential for confounding, because, in every patient, HD prescription was identical between the two successive HD sessions. In stable ESRD patients (as were those of the present study), haemodynamic conditions either before or after HD are reproducible between HD sessions [66].

Finally, for the reasons explained in the Materials and methods section, plasma levels of ADMA and L-arginine were assayed with different methods. As long as each analyte was consistently assayed with the same technique, however, the validity of comparisons made between the groups as well as within the HD group (Figure 3) should not be affected.

Conclusions

In summary, the present study shows for the first time that endothelial NO-dependent vasodilation in the cutaneous microcirculation is not influenced by the plasma level of ADMA in patients on maintenance HD. These results suggest that mechanisms other than or in addition to inhibition of eNOS by accumulated ADMA are responsible for endothelial dysfunction in these patients. They call into question the concept that the accumulation of ADMA is a causal factor for the increased cardiovascular risk in ESRD patients and suggest that ADMA is more likely to represent just a marker of the highly atherogenic environment caused by CKD.

ACKNOWLEDGEMENTS

We gratefully acknowledge the help provided throughout the study by the nursing staff at the outpatient dialysis

unit. We also thank all of the study participants for their time and patience.

FUNDING

This work was supported by Genzyme Switzerland, and by institutional funds.

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Received 29 October 2008/13 February 2009; accepted 5 March 2009
Published as Immediate Publication 5 March 2009, doi:10.1042/CS20080561