Original Article

Blood content of asymmetric dimethylarginine: new insights into its dysregulation in renal disease

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Abstract

Background. Plasma asymmetric dimethylarginine (ADMA), an endogenous inhibitor of nitric oxide synthase, is significantly elevated in patients with kidney disease and is a potential risk factor for cardiovascular disease. Here, we tested whether human whole blood (WB), as in rodent blood, can accumulate free ADMA and whether this accumulation is a function of disease burden.

Methods. In 16 healthy control subjects (CO), 18 patients with ESRD and 18 matched hypertensive patients with normal renal function (HTN), we compared using high-pressure liquid chromatography baseline plasma and WB supernatant (WBSUP) ADMA and symmetrical dimethylarginine (SDMA) concentrations and accumulation during a 5-h incubation. We measured protein turnover in incubated WBSUP to determine if proteolytic processes drive ADMA accumulation.

Results. Elevated plasma ADMA was confirmed in ESRD and HTN populations while basal WBSUP ADMA was significantly higher in ESRD subjects than controls ($P = 0.05$ versus CO; $P = 0.02$ versus HTN). Plasma SDMA followed a similar pattern. Incubation of WBSUP resulted in ADMA release from protein-incorporated stores while SDMA was unaffected. ADMA accumulation in ESRD samples was significantly greater than that in HTN ($P = 0.03$). CO and HTN men showed significantly greater ADMA accumulation than women ($P = 0.01$ and $P = 0.003$, respectively) but no gender difference was observed in the ESRD group ($P = 0.26$). ADMA accumulation correlated with *ex vivo* protein turnover ($R = 0.76$, $P < 0.0001$).

Conclusions. Human blood is capable of releasing physiologically significant quantities of ADMA via proteolytic pathways. Dysregulated ADMA release from WB reservoirs may contribute to the distinctly high plasma ADMA levels in ESRD populations.

Keywords: asymmetric dimethylarginine; cardiovascular risk; end-stage renal disease; symmetric dimethylarginine; whole blood

Introduction

Endothelial dysfunction is present in patients with kidney disease and implicated in accelerated atherosclerosis [1–7]. An endogenous nitric oxide synthase (NOS) inhibitor, asymmetrical dimethylarginine (ADMA), reaches pathophysiologic levels in the plasma of patients with chronic kidney disease [8,9] and is associated with adverse cardiovascular outcomes [9–13]. However, the mechanisms governing the elevation of plasma ADMA are not well understood. Even less is known about its structural isomer, symmetrical dimethylarginine (SDMA), which does not affect NOS activity but may indirectly affect NO synthesis by inhibiting cellular uptake of the NO precursor, L-arginine [14].

Until recently, increased plasma ADMA in patients with nephropathy was believed to result from diminished renal clearance. However, new evidence from our laboratory [15,16] and others [12,17] suggests that more complex mechanisms are responsible for elevations in plasma ADMA. Potentially, enhanced release of ADMA from protein stores via proteolytic pathways [15,18] and/or diminished activity of dimethylarginine dimethylaminohydrolase (DDAH), the only known enzyme to metabolize ADMA [19–21], could lead to increased concentrations.

Using a rodent model, we recently reported that *ex vivo* incubated whole blood (WB) accumulates both ADMA and SDMA [15]. This ADMA accumulation is the result of a balance between release from significant stores of ADMA-containing proteins (nearly 40-fold greater than the amount found free in plasma) via WB proteolytic machinery and ongoing ADMA hydrolysis by DDAH. This WB capacity is substantial enough to represent a potentially major control point for plasma ADMA levels. To our knowledge,

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Fig. 1. Scheme of methods for assessment of ADMA. (A) Plasma is separated from blood and then analyzed for ADMA and SDMA; (B) WB is lysed by freezing and thawing followed by separation of the supernatant, incubation of samples and ADMA analysis; (C) WBSUP is acid hydrolyzed to free all amino-acids, including ADMA, for subsequent determination of total (free plus protein incorporated) ADMA. SPE = solid phase extraction.

there are no published clinical studies assessing total (free and protein incorporated) dimethylarginine (DMA) levels in human WB, nor are there any conclusive reports of altered DMA formation and/or elimination pathways in the WB of patients with kidney disease. Therefore, the goal of this study was to characterize WB ADMA content in three populations with variable plasma levels and cardiovascular risk as a fundamental step to exploring WB ADMA regulatory mechanisms.

Materials and methods

Subject populations

This study was approved by the University of Michigan IRB. After written informed consent, three groups of men and women were enrolled into this study: (1) 16 healthy control subjects (CO); (2) 18 subjects with end-stage renal disease (ESRD) on outpatient chronic hemodialysis (three times/week) at a Michigan Dialysis, LLC unit for at least 1 year prior to enrollment; (3) 18 hypertensive subjects with normal renal function (HTN) matched for age, gender, ethnicity and diabetes status to the ESRD subjects. CO subjects had normal blood pressures ($\leq 130/80$ mmHg), no history of chronic illnesses and were not taking medications. These CO subjects were selected as a reference population to represent normal physiologic DMA characteristics in WB and were not matched to other groups. The HTN group was chosen to control for comorbidities in the ESRD group that could result in altered DMA WB regulation unrelated to renal disease. Normal renal function in the control groups was based on serum creatinine levels ≤ 1.0 and ≤ 1.3 for women and men, respectively. Patients were excluded if they were pregnant, lactating, had a history of hemolytic disease (e.g. sickle cell disease), had hemoglobin levels < 8 g/dL or had clinical signs of acute illness. Medical records were reviewed for inclusion/exclusion criteria, medical history and current medication usage.

Blood samples

Complete blood counts and chemistries were performed by standard clinical laboratory procedures at the University

of Michigan Clinical Pathology Laboratory. Blood samples, immediately chilled and stored for further processing, were drawn from dialysis circuitry immediately prior to the hemodialysis procedure in ESRD subjects and by phlebotomy in CO and HTN groups.

Blood processing and incubation

WBSUP DMA levels are a combination of DMA in plasma and in circulating cells, predominantly the red blood cells. We used an *ex vivo* WB supernatant (WBSUP) incubation protocol previously described [15] and also illustrated in Figure 1. After separation of plasma, the remaining sample was lysed by three freeze-thaw cycles (Figure 1B) and all samples were stored at -20°C until further testing. This technique has been documented to produce $>95\%$ lysis of RBCs [22]. In preliminary studies performed by our group, complete lysis was confirmed with human samples by the absence of RBCs in the supernatant, as visualized under confocal microscopic evaluation, and the absence of hemoglobin in the pellets. In this study, visualization of centrifuged sample pellets confirmed complete lysis. The lysate was centrifuged ($22\,000\text{ g}$ for 8 min at 4°C). $700\text{ }\mu\text{L}$ of WBSUP was aliquotted into a coated (Sigma, SL-2, Sigma-Aldrich, St Louis, MO, USA) 25 mL Erlenmeyer flask and incubated at 37°C in a shaker-water bath for 5 h. Duplicate $50\text{ }\mu\text{L}$ samples were taken at time 0, 0.5, 1, 3 and 5 h for analysis by HPLC.

ADMA and SDMA analysis

Samples were prepared for DMA HPLC analysis using a modification of the method by Heresztyn *et al.* [23] and quantified by reverse-phase liquid chromatography (Breeze System, Waters) as previously described [15,16]. The average method detection limit (MDL) [24] was calculated from eight replicates of $0.75\text{ }\mu\text{M}$ DMA standard and is $0.19\text{ }\mu\text{M}$ for SDMA and $0.02\text{ }\mu\text{M}$ for ADMA. Inter-assay coefficient of variation was 1.7% and the intra-assay coefficient was 2.8%, determined by comparison of plasma quality controls (four replicates) included within three separate sample sets.

Table 1. Clinical data from subject groups

Variables	CO (N = 16)	ESRD (N = 18)	HTN (N = 18)
Age (years)	31 ± 2	60 ± 2	60 ± 2
Gender (M/F)	8/8	10/8	10/8
African American (N)	4	8	8
History of diabetes (N)	0	11	11
History of hypertension (N)	0	18	18
Mean blood pressure (mmHg) ^a	84 ± 2	87 ± 4	85 ± 3
Serum creatinine (mg/dL)	0.8 ± 0.0	NA ^b	0.9 ± 0.0
Estimated glomerular filtration rate ^c	102 ± 10	NA	78 ± 6 ^d
Hemoglobin (g/dL)	14.3 ± 1.4	12.5 ± 0.2	12.9 ± 0.4
Class of medications			
Statins (N)	0	10	12
Metformin (N)	0	0	5*
Thyroxine replacement	0	3	5
Aspirin (N)	0	10	11
ACEI and/or ARB (N)	0	12	12
Erythropoietin (N)	0	11**	0

Comparisons are between ESRD and HTN subjects.

* $P \leq 0.05$.

** $P \leq 0.01$.

^aPost-hemodialysis blood pressure for the ESRD group.

^bSerum creatinine concentrations do not represent renal function in ESRD populations.

^cEstimated glomerular filtration rates are derived from the Cockcroft–Gault equation.

^d $P \leq 0.05$ in comparison between the HTN and CO group.

ACEI = angiotensin receptor inhibitors; ARB = angiotensin receptor blockers.

RBC cytosol DMA content

Free WBSUP and plasma DMA were measured directly by HPLC. The concentration of free DMA in RBCs was estimated from these measurements and the ratio of the RBC to WB volume (assuming that the total volume of blood was the sum of RBC volume and plasma volume neglecting the minor volume component from WBC and platelets). Therefore, the following equation was derived to estimate the RBC cytosol DMA concentrations:

$$\begin{aligned}
 (\text{RBC DMA } (\propto \mu)) = & [\text{WBSUP volume } (\propto \mu) \\
 & \times \text{WBSUP DMA } (\propto \mu)] \\
 & - [\text{Plasma volume } (\propto \mu) \\
 & \times \text{Plasma DMA } (\propto \mu)] / \\
 & \text{RBC volume } (\propto \mu).
 \end{aligned}$$

Acid hydrolysis of blood proteins

To determine whether ongoing dimethylation of WBSUP proteins was responsible for the observed ADMA accumulation, we measured the amount of total ADMA (free plus protein incorporated) in WBSUP samples at baseline

and after 5-h incubation (Figure 1C). Samples of WBSUP were subjected to strong acid hydrolysis to release ADMA present in intact proteins using the procedure we previously described [15]. An increase in total ADMA would suggest protein arginine methyltransferase (PRMT) activity in excess of DDAH activity while a decrease in total ADMA would suggest the opposite.

Lysine analysis

Based on previous studies in rats [15], we estimated the rates of protein turnover by measuring the change in free lysine from baseline to 5 h of incubation. Lysine was measured by HPLC using methods adapted from our ADMA assay but optimized for lysine analysis [25]. Changes in plasma lysine were interpreted as relative differences in WBSUP proteolytic activity and expressed as $\mu\text{moles lysine released} \cdot \text{min}^{-1} \cdot \text{mL}^{-1}$ WBSUP supernatant.

Statistical analyses

Results are presented as mean $\pm$ standard error. Descriptive data between ESRD and matched HTN were compared using the chi-squared test. Repeated-measures ANOVA with Tukey–Kramer adjustments for multiple comparisons were performed to compare between-group and within-group differences in baseline plasma, WBSUP and RBC cytosol levels controlling for baseline hemoglobin levels. Slopes were derived from WBSUP DMA *ex vivo* incubation data at all time points. Slope comparisons between groups and by gender within groups were compared using a random coefficients model with random intercept and adjustment for baseline hemoglobin. In a subset of subjects from each group, group comparisons of ADMA levels after acid hydrolysis at 0 and 5 h were compared using repeated-measures ANOVA with adjustments for multiple comparisons. Also in this subset, the relationship between change in ADMA and lysine over the 5-h period was determined by the Pearson correlation.

Results

Demographic characteristics

As shown in Table 1, demographic characteristics, comorbid conditions and medications (except for metformin and erythropoietin) were similar in the ESRD and matched HTN subjects. Selected to represent a healthy population, the CO groups, by intent, were not matched with the other groups. We did compare renal function in the CO and HTN groups and found that although serum creatinine levels were similar, estimated mean GFR was significantly lower in the patients with HTN ($P = 0.05$).

ADMA and SDMA levels in plasma and WB

Plasma ADMA concentrations were significantly different by group with highest levels in the ESRD subjects ($0.82 \pm 0.03 \mu\text{M}$; $P < 0.001$ versus HTN, CO), followed by the HTN ($0.60 \pm 0.04 \mu\text{M}$; $P < 0.04$ versus CO) and CO

Fig. 2. Free dimethylarginines in blood components. The figure demonstrates the differences between plasma, WB and estimates of RBC cytosol (A) ADMA and (B) SDMA concentrations by group. Freeze-thaw lysis of WB allows the free DMA within the RBC cytosol to mix with plasma levels, yielding WB supernatant ADMA and SDMA. RBC cytosol concentrations were calculated based on the hematocrit and plasma DMA concentrations (see the Materials and methods section). * $P < 0.0001$ within-group differences in ADMA; # $P < 0.05$ between-group differences in ADMA.

subjects ($0.42 \pm 0.02 \mu\text{M}$) (Figure 2A). WBSUP ADMA levels (combined plasma and RBC cytosolic) were significantly higher in ESRD subjects than in CO and HTN subjects ($P = 0.05$ and $P = 0.02$, respectively). Despite these WBSUP ADMA group differences, the estimated cytosolic content was similar in all groups.

When examining within-group differences in blood measurements, WBSUP ADMA levels and cytosol estimates in the CO group were 62% and 147% higher than in plasma, respectively (both $P < 0.0001$). In contrast, ESRD and HTN subjects had similar levels of ADMA in WB and plasma (Figure 2A).

Similar to ADMA plasma levels, plasma SDMA concentrations were highest in ESRD subjects ($1.98 \pm 0.17 \mu\text{M}$; $P < 0.0001$ versus CO and HTN) (Figure 2B). The HTN group trended toward higher SDMA values compared to CO subjects (0.62 ± 0.08 and $0.17 \pm 0.02 \mu\text{M}$, respectively; $P = 0.16$). Similar to plasma ADMA, WBSUP levels were significantly higher in ESRD subjects than in those with renal function ($P < 0.0001$ versus CO and HTN subjects). Only RBC cytosol estimates between HTN and ESRD groups were significantly different ($P = 0.01$).

Fig. 3. Accumulation of free ADMA in incubated WBSUP. Line graph represents mean ADMA accumulation in ESRD (●) and matched HTN (■) WBSUP samples. The rate of accumulation of ADMA is greater in ESRD than in HTN samples. Slopes were compared using random coefficients and random intercept linear regression modeling.

Unlike ADMA, there were no significant differences between plasma, WBSUP or RBC SDMA values within the groups (Figure 2B).

WBSUP incubation

In all groups, the 5-h WBSUP incubation resulted in substantial increases in WBSUP concentrations of ADMA ($P < 0.0001$). Primarily, the matched groups, and not the CO group, were compared to eliminate potentially confounding age-related differences in ADMA levels and overall accumulation [26,27]. ESRD subjects had significantly greater WBSUP ADMA accumulation than matched HTN subjects even after accounting for the differences in basal ADMA and hemoglobin concentrations ($P = 0.03$) (Figure 3A). Also, slopes representing absolute increases in ADMA were compared among all groups. Mean slopes in the CO, HTN and ESRD groups were 0.53 ± 0.04 , 0.53 ± 0.04 and 0.62 ± 0.04 , respectively, with a significantly greater slope in ESRD compared to CO ($P = 0.04$) and HTN groups ($P = 0.01$), while there was no significant difference between the control groups (CO versus HTN; $P = 0.97$). SDMA concentrations remained constant in all groups during incubation (Figure 3B).

Regarding gender, CO and HTN men (Figure 4, top two panels) showed statistically greater increases ($P = 0.01$ and

Fig. 4. Gender differences in incubated blood ADMA accumulation. Line graph shows mean rates of accumulation among men (open symbols) and women (closed symbols) in CO, HTN and ESRD groups. Error bars represent the standard error. Slopes were compared using random coefficients and random intercept linear regression modeling.

$P = 0.003$, respectively) in ADMA compared to CO and HTN women. However, this gender difference is lost in the ESRD group ($P = 0.26$; Figure 4, bottom panel). Also, men in the ESRD and HTN groups had similar increases in WBSUP ADMA levels ($P = 0.59$) while ESRD women had greater increases than HTN women ($P = 0.03$).

Mechanisms of WBSUP ADMA accumulation

In a subset of all groups, WBSUP was acid hydrolyzed to reduce proteins to their constituent amino-acids. The total amount of ADMA (combination of free and protein incorporated) did not change over the 5-h incubation in any group (Figure 5). In contrast, WBSUP lysine accumulation correlated with free ADMA accumulation in all groups ($R = 0.76$, $P < 0.0001$; Figure 6), indicating that *de novo* protein-incorporated ADMA in WBSUP was not increased, but rather previously formed protein-incorporated ADMA was released by endogenous proteolytic processes.

Discussion

The dynamic assessment of ADMA control in WB has the potential to provide greater insight into the complex dysregulation of ADMA in high cardiovascular risk populations. For the first time in humans, we show that there

Fig. 5. Total ADMA in WB supernatant at baseline and after 5-h incubation. Samples of baseline and 5-h-incubated WBSUP were acid hydrolyzed and analyzed for ADMA as described in the Materials and methods section. CO ($N = 6$), HTN ($N = 10$) and ESRD ($N = 13$). No significant difference in baseline total WBSUP ADMA was seen between the three groups or over 5 h of incubation.

Fig. 6. Protein turnover in incubated WBSUP as indicated by lysine correlates with increased ADMA. The accumulation of free lysine during 5-h incubations of WBSUP was measured as a surrogate of total protein turnover. Open and closed symbols represent females and males, respectively. CO = $\circ$; ESRD = $\star$; HTN = $\square$.

is a significant potential source of circulating ADMA that can be released from blood proteins and may be, in part, controlled by protein turnover. Further, we provide new evidence to support dysregulation of ADMA in the blood of ESRD patients including altered ADMA compartmentalization within blood components and increased ADMA accumulation capacity that, unlike controls, exhibits no gender-based differences. These findings highlight the possibility that unknown pathways within blood are involved in the regulation of circulating ADMA and are defective in the ESRD population, thus contributing to the uniquely high plasma ADMA levels in these patients.

Plasma ADMA and SDMA levels were highest in ESRD subjects (Figure 2A and B), confirming numerous previous reports regarding elevated plasma DMA concentrations in patients with renal disease [8,28–31]. SDMA levels in the HTN group are similar to levels observed in other high cardiovascular risk groups with mildly impaired renal function [10,32,33]. Basal WBSUP ADMA concentrations, representing the total amount of free plasma and cytosolic ADMA, are significantly higher in ESRD compared to

HTN and CO subjects (Figure 2). We further estimated that RBC cytosolic content is similar in all groups, suggesting that the WBSUP differences among groups are due to differences in plasma ADMA. Although this requires direct measurement for confirmation, a greater cytosol to plasma difference as demonstrated in the CO group ($0.62 \mu\text{M}$) compared to ESRD and matched HTN groups ($0.29 \mu\text{M}$ and $0.28 \mu\text{M}$, respectively) suggests that regulatory mechanisms exist under healthy conditions. Also, age-dependent regulatory factors may play a role and need further exploration [26,27]. Interestingly, SDMA levels are comparable in these blood compartments in each group, highlighting that there are different control mechanisms for the two DMAs in blood.

The possible mechanisms for differences between plasma and intracellular ADMA may be due to a lower rate of intracellular ADMA release or a higher rate of ADMA elimination from the plasma. These data do not allow us to distinguish which mechanism(s) are responsible, and hence we cannot predict defects resulting in higher plasma levels relative to intracellular ADMA in the ESRD and matched HTN groups. However, possible mechanisms include differential expression of cationic amino acid transporters (e.g. γ^+ transporters) that allow transport of L-arginine and its analogs [14]. Such transporters are present in RBC membranes and are more highly expressed in ESRD subjects [34]; if a higher rate of RBC ADMA accumulation occurs in this population, ADMA efflux may thus be more efficient whereas plasma elimination cannot keep up with the burden. Further studies are required to evaluate the possible role of cationic amino acid transporters in these processes and isolate mechanisms that may be defective in high cardiovascular risk populations that account for higher plasma to cytosol levels of ADMA. Nonetheless, observations demonstrating pathophysiologic elevations of ADMA in hemolytic disease states in humans support our results identifying an enriched intracellular source of ADMA in blood [35]. The increases in ADMA from intracellular sources may be potentially great enough to have a significant effect on NOS pathways.

DMAs are formed by dimethylation of L-arginine residues within intact proteins and released by proteolytic degradation. More is known about type 1 PRMTs that form ADMA than type 2 PRMTs that yield SDMA. However, regulation of these post-translational modifications in humans is unclear [36,37]. One emerging difference between type 1 and type 2 PRMTs is that type 1 appears to be ubiquitous resulting in methylation of multiple proteins while type 2 is more specific and methylation may be cell-cycle dependent [36,37]. Subsequent to methylation, DMAs are liberated via proteolytic degradation and, for the most part, free ADMA is enzymatically degraded to citrulline while free SDMA is excreted in the urine. In the control group, ADMA appears to be tightly controlled in the plasma compared to intracellular stores whereas ESRD and matched HTN subjects have similar values in these compartments (Figure 2A and B). In contrast, SDMA levels are not significantly different in plasma and WB in all groups and, therefore, highlight the importance of renal function in the control of plasma SDMA. The net accumulation of ADMA compared to the unaffected SDMA levels with WB incuba-

tion further highlights the divergence in metabolic fate of these irreversibly methylated proteins.

In our previous report on rat WB [15], we identified a substantial reservoir of protein-incorporated ADMA that could be released by the action of endogenous WB proteases. Here, we extend these findings to human populations with and without pathophysiologic levels in plasma as an initial step to characterize DMA stores in circulation. In support of these findings, other groups have reported a correlation between elevated plasma ADMA levels in conditions of high protein turnover such as in nephrotic syndrome [38], metabolic syndrome [18] and muscular dystrophy [39]. In our study, the ESRD population, known to have the highest levels of plasma ADMA, demonstrated the capacity to accumulate ADMA in greater amounts and at faster rates than similar subjects without renal impairment ($P = 0.03$), suggesting a source for an ongoing ADMA burden. Further, the potential release of ADMA from red cell hemolysis during shear stress associated with hemodialysis may explain why dialytic removal of ADMA appears to be less than predicted based on the size of this molecule [40].

Since many pharmaceutical agents have been reported to affect ADMA plasma levels, we questioned whether differences in ADMA accumulation could have emanated from differential use of medications among the populations (Table 1). Except for erythropoietin and metformin (discussed below), the medications used by the ESRD and HTN populations with the potential for affecting plasma ADMA concentrations include statins [41], thyroxine replacement therapy [42], aspirin [43], angiotensin-converting enzyme inhibitors (ACEI) and angiotensin II receptor blockers (ARB) [44–47]. Usage of the latter medications is balanced between these groups, and thus unlikely to exert differential effects on ADMA accumulation.

Having characterized ADMA accumulation in human WBSUP, we then sought to dissect the mechanistic pathways involved. ADMA release in these samples may involve *de novo* production via dimethylation of L-arginine residues within intact proteins by type 1 PRMTs. The acid hydrolysis data (Figure 5) show no significant total ADMA accumulation during incubation, strongly suggesting that no new ADMA is being formed but rather that a significant reservoir of pre-formed protein-incorporated ADMA exists in human WB. While it is possible that DDAH activity exactly matched new ADMA formation (via PRMT activity) and release, it seems more likely that incubation-induced increases in free ADMA reflect ADMA's release from protein-incorporated stores by the action of endogenous blood proteases. Figure 6 illustrates the simultaneous incubation-induced release of free lysine, which can be considered an independent marker of protein breakdown. Because incubation is performed *ex vivo*, it eliminates hepatic or excretory components from these changes. Thus, we interpret the strong correlation between free lysine and free ADMA levels (Figure 6) as evidence for a common proteolytic pathway for both lysine and ADMA increases. Further studies examining proteolytic enzyme activity or content with respect to ADMA accumulation are needed to confirm these results.

An unexpected finding of this study is that incubated WBSUP from men accumulates significantly more ADMA

than women in control populations but not in ESRD subjects (Figure 4). Thus, the separation between the ESRD and the HTN groups may be completely attributable to gender differences that are lost in ESRD. Indeed when gender is taken into consideration, it appears that significant differences in ADMA accumulation in ESRD and matched HTN groups are due to concentrations in women. The physiological meaning and cause of these gender-related observations are currently unclear. However, the different rates of ADMA accumulation in controls may be due to gender-based differences in protein turnover, a possibility supported by the observation that protein turnover rates in men are generally greater than in women [48]. It is possible that loss of renal function promotes activation of proteolytic processes in women to levels found in men. Finally, the differences in ADMA accumulation between ESRD and HTN women are not likely to be related to estrogen as both groups are in the post-menopausal state and not on estrogen replacement therapy.

There are several limitations in this study. First, there is a disproportionate use of the two reported ADMA-modifying drugs, erythropoietin and metformin. Large doses or prolonged use of erythropoietin analogs may alter plasma ADMA levels via DDAH inhibition [49,50] or upregulation [51] and therefore may be contributing to the differences in plasma and WB ADMA concentrations between ESRD and matched HTN subjects. Metformin utilizes the same membrane transporter as ADMA [52] and thus may affect ADMA clearance. In our study, baseline ADMA and WBSUP ADMA accumulation data did not differ between those who were and were not taking metformin (data not shown), suggesting no compelling influence of this medication on WBSUP ADMA accumulation. Secondly, we did not measure WB DDAH activity, which has been reported to be present in human WB elements [19]. Determination of DDAH activity would improve our understanding of ADMA dysregulation in humans. New techniques to measure DDAH activity in humans are required to characterize the effects of oxidative stress and medications, like erythropoietin on ADMA metabolism. However, in our study protein turnover alone appears to account for the observed increases in ADMA. Thirdly, techniques to measure RBC ADMA concentrations directly were not performed and could be used to confirm our findings. Leukocytes and platelets comprise ~2.5% of the total blood volume and have been shown recently to produce ADMA [53]. Thus, we currently cannot rule out a minor contribution of these blood components to the measured WB ADMA content in our study. Nonetheless, our data in this and a previous study [54] demonstrate that there is potentially a major source of ADMA present in the bloodstream.

Finally, regarding observed gender differences, multiple factors could be responsible for increased protein turnover in the ESRD group unrelated to gender (e.g. infection, inflammation) that may not have adequately been screened in some women and may account for the lack of gender differences in this group. We admit that our study population is small and further studies are required to examine the links between protein turnover, gender and ADMA release in ESRD. Nonetheless, by this assessment of total store

(free plus protein incorporated) of ADMA in circulation and the *ex vivo* capacity of blood to release and accumulate free ADMA, we begin to uncover new dynamic processes that may significantly contribute to the control of this endogenous NOS inhibitor. Potentially, further clarification of these mechanisms and defects defining the ADMA burden in renal disease will help improve our understanding of this putative contributor to atherosclerotic disease processes and aid in the identification of novel therapeutic strategies for populations at high risk of cardiovascular disease.

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Conflict of interest statement. None declared.

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