Reduced Renal Methylarginine Metabolism Protects against Progressive Kidney Damage

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ABSTRACT

Nitric oxide (NO) production is diminished in many patients with cardiovascular and renal disease. Asymmetric dimethylarginine (ADMA) is an endogenous inhibitor of NO synthesis, and elevated plasma levels of ADMA are associated with poor outcomes. Dimethylarginine dimethylaminohydrolase-1 (DDAH1) is a methylarginine-metabolizing enzyme that reduces ADMA levels. We reported previously that a DDAH1 gene variant associated with increased renal DDAH1 mRNA transcription and lower plasma ADMA levels, but counterintuitively, a steeper rate of renal function decline. Here, we test the hypothesis that reduced renal-specific ADMA metabolism protects against progressive renal damage. Renal DDAH1 is expressed predominately within the proximal tubule. A novel proximal tubule–specific Ddah1 knockout (Ddah1PT−/−) mouse demonstrated tubular cell accumulation of ADMA and lower NO concentrations, but unaltered plasma ADMA concentrations. Ddah1PT−/− mice were protected from reduced kidney tissue mass, collagen deposition, and pro-fibrotic cytokine expression in two independent renal injury models: folate nephropathy and unilateral ureteric obstruction. Furthermore, a study of two independent kidney transplant cohorts revealed higher levels of human renal allograft methylarginine-metabolizing enzyme gene expression associated with steeper function decline. We also report an association among DDAH1 expression, NO activity, and uromodulin expression supported by data from both animal and human studies, raising the possibility that kidney DDAH1 expression exacerbates renal injury through uromodulin-related mechanisms. Together, these data demonstrate that reduced renal tubular ADMA metabolism protects against progressive kidney function decline. Thus, circulating ADMA may be an imprecise marker of renal methylarginine metabolism, and therapeutic ADMA reduction may even be deleterious to kidney function.


CKD poses an increasing global disease burden and contributes to cardiovascular disease (CVD), which is the leading cause of death worldwide.1,2 CKD is associated with reduced bioavailability of nitric oxide (NO), which is required to maintain normal vascular and kidney function.3,4 Asymmetric dimethylarginine (ADMA) is released into the cytoplasm during normal protein turnover and competes with L-arginine binding at the active site of nitric oxide synthase (NOS) to block NO synthesis. The methylarginine-metabolizing enzymes (MAMEs)—dimethylarginine dimethylaminohydrolase isoforms 1 and 2 (DDAH 1 and 2) and alanine-glyoxylate aminotransferase-2

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(AGXT2)\textsuperscript{5}—metabolize ADMA and other methylarginines to inactive products. Thus, MAMEs represent alternative pathways of endogenous NO regulation.\textsuperscript{6} The kidney expresses all MAME and NOS isoforms in a cell-specific distribution,\textsuperscript{5,7,8} thereby providing the means for intricate, cell-type-specific control of NO bioavailability. Renal NO regulates hemodynamics,\textsuperscript{9} afferent arteriolar vascular reactivity,\textsuperscript{10} glomerular filtration,\textsuperscript{11} tubular reabsorption, and solute excretion.\textsuperscript{8,12} Specifically, the proximal tubule (PT) is the principal renal cell type expressing the enzyme with the greatest activity for metabolism of ADMA, DDAH1;\textsuperscript{7,13} however, how this influences tubular reabsorption and response to injury through NO-ADMA regulation is unclear. Human studies demonstrate arteriovenous ADMA gradients across the kidney, which represent a large proportion of total-body ADMA clearance through enzymatic metabolism and urinary excretion.\textsuperscript{14} Consequently, plasma ADMA levels rise with declining kidney function, as demonstrated by Vallance and colleagues’ early report of elevated plasma ADMA, lower NO, and vascular dysfunction in patients with ESRD.\textsuperscript{15}

Increased circulating ADMA is a powerful predictor of progressive CVD and CKD, independent of eGFR and other traditional risk factors.\textsuperscript{16–20} Because plasma ADMA increases with declining kidney function, the direction of causality is difficult to resolve by correlation alone. Although increased plasma ADMA levels may correlate with reduced total-body NO bioavailability, they do not necessarily reflect the balance of NOS inhibition within the renal interstitium during progressive CKD. Our published data suggest that lower plasma ADMA does not necessarily protect against renal function decline and that increased DDAH1 activity limited to the kidney may in fact be harmful. Thus, we reported a noncoding DDAH1 variant (rs17384213) that was associated with higher renal DDAH1 mRNA expression and lower plasma ADMA concentrations. Counterintuitively, however, this correlated with a faster rate of eGFR decline in two independent cohorts of patients with CKD.\textsuperscript{21} Competitive inhibition of NOS by ADMA may play both protective and pathogenic roles,\textsuperscript{6} determined by such factors as disease site, time course, and magnitude of response.

In light of these uncertainties, it is clearly important to define the role of PT-specific DDAH1 in CKD progression. We hypothesized that reduced renal DDAH1 activity protects against renal function decline, independent of circulating NO and ADMA.

RESULTS

PT-Specific Gene Targeting

The PT is the principal renal cell type to express DDAH1 (\textsuperscript{7,11} and Figure 1). Conditional PT-specific gene manipulation was achieved using the KAP2iCre gene construct.\textsuperscript{22} Regulatory elements of the kidney androgen–regulated protein (KAP) promoter and angiotensinogen genes confer highly PT-specific, codon-optimized Cre (iCre) expression in response to androgen stimulation.

Two double transgenic mouse strains were created, both possessing the KAP2iCre gene along with either ROSA26eYFP (a reporter strain designed to confirm PT-specific gene manipulation [loxP sites flank a YFP STOP codon; Figure 2A]) or floxed Ddah1 (loxP sites flank exon 1 of Ddah1, which encodes the initiating methionine residue and the first 100 amino acids of the protein; Figure 2D).\textsuperscript{23} To achieve temporal control over PT-specific KAP2iCre expression and gene disruption, only female mice were treated with exogenous testosterone and used for study. YFP expression in female KAP2iCre/ROSA26eYFP reporter mice treated with testosterone was demonstrated using ImageStream analysis of isolated renal tubular cells (Figure 2B) and by renal tissue histologic testing (Figure 2C). Mice not possessing the KAP2iCre transgene did not express YFP.

PT-Specific Ddah1 Deletion

The Ddah1 gene was deleted after testosterone exposure in KAP2iCre/Ddah1-floxed mice (Ddah1\textsuperscript{PT/–}) but not in Ddah1-floxed (Ddah1\textsuperscript{B/B}) controls (Figure 2E). Reduced Ddah1 expression was clearly observed in the kidney, where it exceeded 50% (\textit{P}<0.05) (Figure 2F). Nonsignificant reductions were detected in the liver and brain. Whole kidney lysate DDAH1 protein expression in Ddah1\textsuperscript{PT/–} mice was reduced by approximately 80% (\textit{P}<0.001) (Figure 2G) and enzymatic DDAH1 activity was reduced by approximately 70% (\textit{P}<0.05) (Figure 2H).

Downstream Effects of PT-Specific Ddah1 Deletion on Methylarginines and NO

ADMA recovered from isolated renal tubules was almost 7-fold higher (\textit{P}<0.05) and nitrogen oxide species, nitrite NO\textsubscript{2} and nitrate NO\textsubscript{3} (NOx) was 2.5-fold lower (\textit{P}<0.05) in Ddah1\textsuperscript{PT/–} mice versus Ddah1\textsuperscript{B/B} controls (Figure 3, A and B). The effect of PT-specific Ddah1 deletion was restricted to tubular isolates and was not evident in whole kidney tissue, plasma, or urine, indicating tubule-specific ADMA-NO perturbation (Figure 3, A–H). This was further demonstrated by an absence of baseline effects on renal expression of alternative MAME isoforms and NOS enzymes (Figure 3I), systolic BP (Figure 3J), renal function

**Figure 1.** Immunohistochemical staining for DDAH1. Human allograft protocol biopsy tissue. (A) DDAH1 staining is seen in the epithelial cells of the proximal tubule (arrows) and not in the glomerulus (g) or control. (B) DDAH1 antibody preincubated with DDAH1 peptide. Original magnification, ×200.
according to plasma creatinine (Figure 3K), and urinary volumes (Figure 3L).

Effects of PT-Specific Ddah1 Deletion on Urine Biochemistry

Urinary electrolyte, protein, and amino acid concentrations were not significantly affected by PT-specific Ddah1 deletion (Figure 4, A–F). However, through use of a proteomic approach, tubular Ddah1 disruption significantly affected several urinary peptides under baseline conditions. Of 1057 peptides screened in Ddah1PT/−/− and Ddah1fl/fl mouse urine, relative concentrations of 82 peptides were significantly altered (P<0.05) (Figure 5). Most significantly downregulated in Ddah1PT/−/− mouse urine was uromodulin (UMOD) (8.5-fold lower in urine from
Ddah1<sup>PT−/−</sup> mice compared with Ddah1<sup>fl/fl</sup> controls (P, 0.001 or q=0.00083) (Figure 5). In addition, urinary Col1α1 was downregulated 6.5-fold in Ddah1<sup>PT−/−</sup> mice (P, 0.001; q=0.0017) at baseline.

**Effect of PT-Specific Ddah1 Deletion in Folate Nephropathy**

A single intraperitoneal injection of folate induced acute tubular injury in all mice, manifested by gross tubular dilatation, luminal casts, and interstitial inflammatory cell infiltrates (Figure 6; see Supplemental Material for a full characterization of folate nephropathy in wild-type mice). At 12 weeks after folate administration, significant differences emerged between Ddah1<sup>PT−/−</sup> and Ddah1<sup>fl/fl</sup> control mice. Renal collagen deposition was significantly lower in Ddah1<sup>PT−/−</sup> mice treated with folate than in Ddah1<sup>fl/fl</sup> controls (P<0.05) (Figures 7 and 8). In addition, reduction in renal mass according to kidney-to-body weight ratios was significantly attenuated in Ddah1<sup>PT−/−</sup> versus Ddah1<sup>fl/fl</sup> controls (P<0.05; q=0.0003) (Figure 8).

**Figure 3. Effects of PT-specific Ddah1 deletion on ADMA and NO are tubule-specific.** (A and B) Tubular cells isolated from Ddah1<sup>PT−/−</sup> mice contained significantly higher ADMA and lower NO concentrations. SDMA, a methylarginine that is not a substrate for DDAH1, was unaffected. (C–H) These changes were not evident in whole kidney tissue, plasma, or urine. (I) Tubular cell expression of other MAMEs and NOS isoforms (neuronal, inducible, and endothelial) was unaffected in Ddah1<sup>PT−/−</sup> mice. (J–L) Similarly, systolic BP, plasma creatinine, and urinary volumes were not altered. Mann–Whitney U test, n=6 each group; *P<0.05. (Note: The measurement of urinary NOx is not necessarily an accurate determination of renal NOS activity, particularly in the context of a nitrate-containing diet). ns, not significant. Error bars, mean±SEM.
mice (P<0.01) (Figure 7B). Kidney tissue profibrotic cytokine expression was 3- to 5-fold lower in Ddah1\textsuperscript{PT−/−} mice (Col1a2: P<0.05; TGF-β: P<0.01; endothelin-1: P<0.05) (Figure 7C).

Furthermore, at 12 weeks, Ddah1\textsuperscript{PT−/−} mice were protected from other manifestations of advanced renal impairment, such as hypertension (systolic BP; P<0.01) (Figure 7D) and raised

Figure 4. Effects of PT-specific Ddah1 deletion on urinary electrolytes, protein, and amino acids at baseline. No consistent changes in urine biochemistry were identified in Ddah1\textsuperscript{PT−/−} mice including concentrations of sodium (A), phosphate (B), glucose (C), protein (D); according to urine protein:creatinine ratio [UPCR] or creatinine (F). (E) Amino acids l-citrulline and l-arginine were product and substrate for NO synthesis, respectively; l-glutamine was selected because of its abundance in urine and has been shown to be elevated in patients with tubular dysfunction (Fanconi syndrome).\textsuperscript{51} All values corrected for creatinine concentration. Mann–Whitney U test, n=8 (Ddah1\textsuperscript{fl/fl}) and n=20 (Ddah1\textsuperscript{PT−/−}). ns, not significant; KC, KAPiCre. Error bars, mean±SEM.

Figure 5. Heat map of urinary proteomic analysis after PT-specific Ddah1 deletion at baseline. Eighty-two of 1057 peptides screened were significantly altered by Ddah1 deletion. The 10 most significantly down- and upregulated peptides are represented here. (The q values represent values of significance adjusted for multiple comparisons; n=4.)
plasma creatinine \((P<0.001)\) (Figure 7E). There was a trend toward lower levels of proteinuria in \(Ddah1^{PT-/-}\) mice, although this did not reach statistical significance \((P=0.21)\) (Figure 7E).

**Effect of PT-Specific \(Ddah1\) Deletion in Unilateral Ureteric Obstructive Nephropathy**

To confirm that the protection against fibrosis observed with PT-specific \(Ddah1\) deletion was not a phenomenon unique to folate toxicity, \(Ddah1^{PT-/-}\) mice were exposed to a second renal injury model: unilateral ureteric obstruction (UUO). (See Supplemental Material for a detailed characterization of UUO nephropathy in wild-type mice).

At 2 weeks after UUO, severe tubulointerstitial disease was evident in kidney sections from obstructed kidneys, with gross tubular dilatation, epithelial cell flattening, parenchymal thinning, and collagen deposition (Supplemental Material). Compared with \(Ddah1^{fl/fl}\) controls, kidney tissue from \(Ddah1^{PT-/-}\) mice exhibited reduced loss of renal mass \((P<0.05)\) and collagen deposition \((P<0.05)\) (Figure 9, A–C). Furthermore, kidney tissue expression of profibrotic genes Col1\(\alpha2\) and TGF-\(\beta\) was significantly lower in \(Ddah1^{PT-/-}\) mice \((P<0.05)\) (Figure 9D).

**Allograft MAME Expression and eGFR Decline in Humans**

To confirm the preceding laboratory findings, we measured kidney tissue MAME (DDAH1 and AGXT2) transcript levels and eGFR decline in two independent human renal transplant cohorts (Table 1). In both the London and the Virginia cohort, there was a strong positive correlation between corrected DDAH1 expression and corrected AGXT2 expression (Supplemental Material). Therefore, for further analysis of eGFR decline we used a composite methylarginine gene expression calculated from the geometric mean of the DDAH1 and AGXT2 expression. In both cohorts, higher MAME expression was associated with a steeper decline in eGFR during the 1 year after allograft biopsy (Table 2). Although there was an association between MAME expression and baseline eGFR in the Virginia cohort, this did not reach statistical significance in the London participants. The multilevel approach allows estimation of the association between gene expression and eGFR decline independent of the baseline eGFR, as illustrated in Figure 10.

**Association among Kidney DDAH1 Expression, NO Activity, and UMOD**

Given the findings of lower urinary UMOD peptide in proteomic analysis of \(Ddah1^{PT-/-}\) mouse urine, we examined the association between DDAH1 gene expression and urinary UMOD protein concentration in the London patient cohort. Although there was only a trend toward a positive correlation in the cohort as a whole, DDAH1 gene expression and urinary UMOD were significantly associated when the analysis was restricted to recipients of live-donor allografts (to minimize
the effect of ischemia and other confounding variables associated with deceased donor organs) \( (P=0.039) \) (Figure 11A).

To establish whether the positive correlation between renal DDAH1 activity and UMOD protein expression observed in the \( \text{Ddah1}^{\text{PT}^{+/-}} \) mouse and human cohort was NO dependent, we performed a further \textit{in vivo} study in wild-type mice receiving a nonselective NOS inhibitor (L-NG-nitroarginine methyl ester [L-NAME]). Changes in renal UMOD protein expression were assessed. After 2 weeks of L-NAME treatment (1 mg/ml dissolved in drinking water; concentrations previously established to systemically inhibit NOS \textit{in vivo} \( 24-26 \)), kidney tissue NOx (nitrites and nitrates) were reduced by 25% \( (P<0.01) \). Furthermore, UMOD protein expression was significantly reduced after L-NAME treatment: 40% in kidney tissue \( (P<0.01) \) and 30% in urine \( (P<0.01) \) (Figure 11, B–E).

**DISCUSSION**

This study focused on MAME activity limited to kidney tissue by exposing a novel transgenic mouse strain to two different forms of renal injury and examining renal biopsy and outcome data from two human cohorts. We conclude that local imbalances of the NO-ADMA-MAME axis may determine kidney function decline more reliably than circulating ADMA. PT cell–specific \( \text{Ddah1}^{\text{PT}^{+/-}} \) deletion was dissociated from baseline plasma ADMA and systemic BP. The observed protection against kidney collagen deposition and functional decline was therefore independent of systemic confounders and distinguishes a critical difference between local (protective) and systemic (harmful) effects of ADMA in renal function decline.

Data from two human renal transplant cohorts support the animal studies. Modeling renal function over time in the posttransplant period using laboratory values is not straightforward. Our estimates show substantial unexplained variation that was not significantly improved with addition of other clinical variables. Despite this, we detected an association between eGFR decline and increased MAME expression in both renal transplant cohorts. The relationship between the DDAH1 and \( \text{AGXT2} \) enzymes is unclear, but their relative expression was strongly correlated. A summary composite (geometric mean) of both the enzymes was used to demonstrate that raised kidney tissue MAME expression correlated with poorer outcomes in terms of eGFR.

**Figure 7.** Effects of PT-specific \( \text{Ddah1} \) deletion at 12 weeks after folate administration. (A) Renal collagen deposition was significantly reduced in \( \text{Ddah1}^{\text{PT}^{+/-}} \) mice versus \( \text{Ddah1}^{\text{fl}^{+/-}} \) controls (4.5 versus 7.2%; \( P<0.05 \)). Two-way ANOVA with Bonferroni post-test, \( \ast P<0.05 \). (B) \( \text{Ddah1}^{\text{PT}^{+/-}} \) mice had significantly attenuated kidney-to-body weight ratio reduction compared with \( \text{Ddah1}^{\text{fl}^{+/-}} \) controls (\( P<0.001 \); values are deviations from means of vehicle-treated controls). (C) Quantitative RT-PCR analysis revealed significantly lower renal tissue profibrotic cytokine expression in \( \text{Ddah1}^{\text{PT}^{+/-}} \) mice. Colla2 was decreased >3-fold \( (P<0.05) \), TGFβ was decreased >5-fold \( (P<0.01) \), and endothelin-1 (ET-1) was decreased almost 3-fold \( (P<0.05) \). (D and E) Systolic BP and serum creatinine were lower in \( \text{Ddah1}^{\text{PT}^{+/-}} \) mice; \( \ast \ast P<0.01 \) and \( \ast \ast \ast P<0.001 \), respectively. (F) Proteinuria according to urine protein-to-creatinine ratio (UPCR) was reduced in \( \text{Ddah1}^{\text{PT}^{+/-}} \) mice, but this change was not statistically significant. Mann–Whitney \( U \) test unless otherwise stated, \( n=9 \) (\( \text{Ddah1}^{\text{fl}^{+/-}} \)) and \( n=12 \) (\( \text{Ddah1}^{\text{PT}^{+/-}} \)). \( \ast P<0.05 \); \( \ast \ast P<0.01 \); \( \ast \ast \ast P<0.001 \). ns, not significant. Error bars, mean±SEM.
Reduced renal collagen deposition in PT-specific Dda1 deleted mice at 12 weeks after folate administration. Kidney sections were stained with picrosirius red and viewed through circularly polarized light. Images (original magnification, ×25) were processed using ImageJ software. Images were stitched to produce a complete kidney section image, total section area was defined by texture thresholding, and collagen (yellow) was quantified as a percentage of total kidney section area. n=3 replicates (i–iii) shown for representation of n=9 (Dda1fl/fl) and n=12 (Dda1PT−/−).

Our findings suggest a reanalysis of previously published human observational data showing that elevated plasma ADMA was associated with progression of renal disease.19,20,27 Interestingly, the largest study of patients with CKD to date found no association between serum methylarginine levels and renal progression once baseline GFR was considered.19 Previously, using a Mendelian randomization approach (with less susceptibility to reverse causation and residual confounding), we reported a gene variant that conferred higher kidney tissue DDAH1 mRNA expression and was associated with more rapid renal decline.21

Previously published rodent studies of kidney fibrosis have reported that DDAH1 and NO are protective while ADMA is pathogenic; however, significant study design issues limit their interpretation. ADMA or L-NAME infusion into mice exacerbated renal fibrosis, but both raised SBP by approximately 60 mmHg, suggesting hypertensive injury28; global DDAH1 overexpression, however, protected against fibrosis in angiotensin and surgical nephron-reduction models of CKD.29,30 Genetic DDAH1 overexpression decreases circulating ADMA and introduces unmeasured effects upon normal regulatory mechanisms. Furthermore, DDAH1 overexpression occurs indiscriminately in cell types that do not normally express DDAH1, but play significant roles in inflammation and fibrosis (e.g., macrophages). Here, PT-specific Dda1 gene deletion raises ADMA and reduces NO availability only within tubules and provides protection against fibrosis and functional decline in two different models of nephropathy.

Proteomic urinalysis data in Dda1PT−/− mice suggests that reduced tubular NO activity may confer protection against progressive fibrosis in folate and UUO nephropathy through reduced expression of collagen and UMOD proteins. A stimulatory role of NO activity upon collagen deposition and wound-healing in skin and tendon tissue has been shown in in vitro and in vivo studies using exogenous NO donors,31 iNOS overexpression32 and even dietary L-arginine supplementation.33 Published literature confirms NO as a pleiotropic molecule in the kidney as well as other systems34 with both protective and deleterious effects exerted by NO upon collagen deposition and fibrosis in renal disease.35–39 What is clear is that maladaptive, pro-fibrotic responses to injury are, at least in part, driven by cross-talk between high NO activity and fibrotic mediators such as TGF-β and matrix metalloproteases through SMAD signaling and other pathways.37 Relevant to our own findings, pharmacologic DDAH1 inhibition (causing reduced NO activity) has been demonstrated to reduce collagen deposition in a TGF-β and SMAD-dependent manner in a bleomycin model of pulmonary fibrosis.40

An association between urinary UMOD and renal DDAH1 expression was identified in both the Dda1PT−/− mouse and human cohort data. An 8.5-fold reduction of UMOD in Dda1PT−/− suggests that functional changes in MAMEs and NO synthesis within the proximal tubule are communicated downstream along the nephron to the thick ascending limb, where UMOD is exclusively expressed.41 A link between NO activity and UMOD expression was confirmed in wild-type mice exposed to a nonselective NOS inhibitor (L-NAME), which resulted in reduced UMOD protein expression in kidney tissue and urine. Of note, NO exerts regulatory control over two transcription factors—nuclear factor of activated T cells42 and Oct-143—both of which have consensus-binding sites in the promoter region of UMOD.44 These observations raise the possibility of direct regulation of UMOD expression by NO, a suggestion that certainly warrants further study. Genome-wide association studies identify many UMOD polymorphisms that associate with
UMOD expression, hypertension, CKD, and kidney function. A recent report confirmed that common UMOD gene variants increase UMOD expression and that (salt-sensitive) hypertension and CKD progression may be induced through NKCC2 cotransporter stimulation. In our study, reduced Ddah1 metabolism of methylarginines (in the PT) correlates with lower UMOD expression downstream and, consistent with published data, may in part explain the protective effect observed against kidney disease progression.

This study has several limitations. A minor degree of extrarenal (liver and brain) Ddah1 gene deletion in the Ddah1 PT−/− mouse was suggested using sensitive PCR-based assays, although this did

Figure 9. Effects of PT-specific Ddah1 at 2 weeks after UUO. (A and B) Renal collagen deposition was significantly lower than in Ddah1 fl/fl controls (determined by nonbiased software analysis of picrosirius red–stained sections; 6.6% versus 10.4%; P<0.05). (C) Renal parenchyma was less contracted in Ddah1 PT−/− mice (assessed by percentage reduction of whole kidney section area [compared with unobstructed contralateral kidney in same mouse]; 35% versus 47% in Ddah1 fl/fl controls; P<0.05). (D) Quantitative RT-PCR analysis revealed significantly lower renal tissue profibrotic cytokine expression in Ddah1 PT−/− mice. Col1α2 was decreased >4-fold (0.07 versus 0.29 AU in Ddah1 fl/fl controls; P<0.05); TGFβ was decreased 2-fold (0.19 versus 0.37 AU in Ddah1 fl/fl controls; P<0.05); and endothelin-1 was decreased approximately 40% (0.22 versus 0.35 AU; P=NS). Mann–Whitney U test, n=13 (Ddah1 fl/fl) and n=20 (Ddah1 PT−/−). *P<0.05. ns, not significant. Error bars, mean±SEM.
Table 1. Demographic characteristics of two human renal transplant cohorts

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<th>London Cohort</th>
<th>Virginia Cohort</th>
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<tr>
<td>Women, n (%)</td>
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<td>14 (38.9)</td>
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<tr>
<td>Median age (yr)</td>
<td>47 (40–58)</td>
<td>55 (44.5–61)</td>
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<td>Ethnicity, n (%)</td>
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<td>Black</td>
<td>13 (35.14)</td>
<td>7 (19.4)</td>
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<td>Other</td>
<td>3 (8.11)</td>
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<tr>
<td>White</td>
<td>17 (45.95)</td>
<td>28 (77.8)</td>
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<td>Live donor, n (%)</td>
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<td>1 (2.8)</td>
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<tr>
<td>Median donor age (yr)</td>
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<td>Delayed graft function, n (%)</td>
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<tr>
<td>Rejection before protocol biopsy, n (%)</td>
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<td>1 (2.8)</td>
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<tr>
<td>Mean eGFR at biopsy (SD)</td>
<td>52.9 ± 17.7</td>
<td>56.2 ± 19</td>
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<td>Median UMOD (μg/mL)</td>
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Table 2. Estimated SDs for eGFR values

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<th>Estimated SDs (95% CI)</th>
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<td>GFR change over time (ml/min per 1.73 m² per d)</td>
<td>0.032 (0.014 to 0.049)</td>
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<td>Increase in GFR at biopsy (ml/min per 1.73 m² per unit MAME)</td>
<td>16.2 (7.3 to 43.4)</td>
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<tr>
<td>Change in GFR over time (ml/min per 1.73 m² per unit MAME per d)</td>
<td>–0.065 (–0.107 to –0.023)</td>
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(1) Between patients at baseline (intercept): London, 16.2 ml/min per 1.73 m² (95% confidence interval [95% CI], 12.8 to 20.6); Virginia, 14.9 ml/min per 1.73 m² (95% CI, 11.4 to 19.5); (2) between patients over time (slope): London, 0.02 ml/min per 1.73 m² per day (95% CI, 0.01 to 0.03); Virginia, 0.03 ml/min per 1.73 m² per day (95% CI, 0.02 to 0.05); (3) within patients: London, 7.1 ml/min per 1.73 m² (95% CI, 6.6 to 7.5); Virginia, 7.3 ml/min per 1.73 m² (95% CI, 6.8 to 7.9). Correlation between baseline eGFR (intercept) and slope: London, 0.06 (95% CI, –0.49 to 0.57); Virginia, 0.17 (95% CI, –0.25 to 0.54). A decline of 0.05 to 0.065 ml/min per 1.73 m² per day per unit MAME may appear trivial, but a 50% higher renal MAME expression would be reflected in an additional annual rate of renal function decline of 9–12.0 ml/min per 1.73 m² (MAME expression units across these cohorts were normalized to values between 0 and 1).

CONCISE METHODS

Animal Studies
Animal experiments and husbandry were performed under United Kingdom Home Office License after approval by the local ethics committee. The KAPiCre transgenic mouse on a C57B6 background was purchased from The Jackson Laboratory (strain name: B6.Cg-Tg(Kap-cre)29066/2Sig/1). The ROSAYFP mouse (mixed background) was a kind gift from Professor Graham Williams (Imperial College, London, UK). Ddah1 floxed mice on a mixed background were previously developed and supplied by our group. LoxP sites flank exon 1 of Ddah1 (exon 1 contains the initiating methionine codon and encodes the first 100 amino acids of DDAH1 [approximately 35% of the total protein]). To achieve temporal activation of KAPiCre, female mice were treated with a subcutaneous testosterone pellet (Innovative Research of America, Sarasota, FL) for a minimum of 10 days. Animals were placed in metabolic cages for a maximum of 24 hours and urine collected was centrifuged at 8000 g for 10 minutes, and the supernatant stored at –80°C.

Invasive BP recording was performed under terminal (1%) isoflurane anesthesia. A Mikro-Tip pressure transducer catheter (Millar Instruments, Houston, TX) was placed into the carotid artery. Recordings were obtained using the Powerlab system with analysis on Chart software (both from ADInstruments, Oxfordshire, UK).

Folte Nephropathy
Mice received a single intraperitoneal injection of folate (240 μg/g) dissolved in 0.3M NaHCO₃ vehicle (20 mg/ml) or vehicle control alone (both from Sigma-Aldrich, Dorset, UK). Mice were euthanized by CO₂ narcosis and cervical dislocation at day 2, day 14, or day 84 (12 weeks). Blood collected by direct cardiac puncture was stored on ice before centrifugation at 8000 g for 10 minutes, and the supernatant stored at –80°C.

UOO Nephropathy
Under isoflurane inhalation anesthesia, mice were shaved, drugged, and positioned on a heat mat. In the supine position, a left flank incision not reach statistical significance in Ddah1 mRNA transcription in these tissues. This may represent a constitutive leak of Cre expression autonomous of androgen stimulation, or Cre activation in response to even very low levels of circulating testosterone in women. It is unlikely that such changes in neuronal or hepatic Ddah1 activity, in the absence of altered BP or systemic ADMA, could influence renal fibrosis. Off-target Cre expression and activity are widely under-reported.47 Cre toxicity causes cardiomyopathy,48 brain development defects,49 and widespread apoptosis in embryos.50 To our knowledge, no studies have described Cre toxicity relating to fibrosis, and any effects to date suggest that Cre disrupts normal cellular function or architecture rather than preserve it, as seen in the Ddah1PT–/– mouse. Other limitations, such as relatively small patient sample numbers, are counterbalanced by findings that are consistent across two very different experimental models of renal injury and human observational data examining gene expression and clinical outcomes in two independent cohorts.

In conclusion, we demonstrate that a reduction in renal ADMA metabolism protects against renal function decline in the context of renal injury. Whether therapeutic reduction of circulating ADMA would improve cardiovascular outcomes at the same time as accelerating renal fibrosis requires further investigation. Our findings suggest that, at least in the kidney, nonselective ADMA reduction could be detrimental.

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exposed the left ureter and was ligated twice with 4–0 nylon sutures and transected. Following suture closure, animals received intramuscular diclofenac analgesia (Vetagesic) and recovered in heated chambers. Mice were euthanized by cervical dislocation, followed by organ harvesting at three time points: 1, 2, and 3 weeks.

**L-NAME Experiment**
Female littermates wild-type C57BL/6 mice aged approximately 10 weeks were randomly allocated to receive plain deionized drinking water or the same water containing dissolved (L-NAME; 1 mg/ml, Enzo Life Sciences). There were eight mice in each group. Water was changed every 3 days and mice were euthanized by CO₂ narcosis and cervical dislocation. Urine was collected for 24 hours before euthanasia; kidney tissue was harvested, snap frozen in liquid nitrogen, and stored at −80°C before further analysis.

**Renal Tubule Isolation**
Mice were euthanized by CO₂ narcosis and cervical dislocation. The abdominal aorta was ligated superior to the renal arteries, and magnetic beads (Dynabeads M-450 Tosylactivated, Invitrogen, Paisley, UK) suspended in ice-cold HBSS were infused via an aortic catheter placed distally. Kidney perfusion was confirmed by rapid Blanching. Kidneys were then dissected out, decapsulated, and minced with a scalpel before incubation in collagenase (1 mg/ml; Sigma-Aldrich) at 37°C for 30 minutes with agitation. The resulting homogenate was successively sieved through 100-micron and 50-micron pore sizes, and fragments caught by the latter were resuspended in cold HBSS. Magnetic separation of glomerular and tubular fragments was confirmed by microscopic examination (bead entrapment visible within glomerular capillary loops). Purified tubules were pelleted by centrifugation at 1000 g and stored at −80°C for future use or subjected to further digestion (Accu-max; protease/ DNAse solution; Innovative Cell Technologies Inc., San Diego, CA) before ImageStream analysis (Amnis Corp., Seattle, WA). Cells were gated according to spherical shape and yellow fluorescence before automated single-cell image capture.

**Biochemical Methods**
The Agilent 6400 Series Triple Quadrupole liquid chromatography-tandem mass spectrometry (LC-MS/MS) system was used for this

![Figure 10](image-url)
study. Biologic compounds optimized for detection included methyl-arginines (Calbiochem, Nottingham, UK), amino acids, and creatinine (the latter two from Sigma-Aldrich). Biologic samples, including plasma, urine, and tissue lysates, were prepared by methanol protein precipitation in a 1:5 dilution; the samples were vortexed and the pellet removed by centrifugation. Sample solutions were evaporated to dry in a heat-block and resuspended in mobile phase (0.1% formic acid). An internal standard was added (7-deuterated ADMA; Cambridge Isotope Laboratories, Tewksbury, MA) before precipitation to allow correction for extraction efficiency and ion suppression at the point of detection (ions in large abundance can outcompete minority ions at the detector). A hypercarb (Thermo Fisher Scientific) chromatography column was used, and the mobile phase consisted of 0.1% formic acid, 1% acetonitrile (increasing to 50% between 5 and 10 minutes), over a total run time of 15 minutes. Data were collected using Agilent (Berkshire, UK) MassHunter data acquisition software and analyzed with MassHunter qualitative analysis software before downloading of raw data into Excel (Microsoft Corp., Redmond, WA) for processing. The mass spectrum iterative analysis software before downloading of raw data into Excel (Microsoft Corp., Redmond, WA) for processing. The mass spectrum variables were as follows: ADMA, mass-to-charge ratio (m/z): 203.3–46.0; collision energy (CE): 12; symmetrical dimethylarginine, m/z: 203.3–70.2, CE: 24; arginine, m/z: 175.2–60.1, CE: 8; citrulline, m/z: 176.2–86.2, CE: 16; glutamine, m/z: 147.0–56.3, CE: 0; d7-ADMA, m/z: 210.0–46.0, CE: 24; creatinine m/z: 114.0–44.0, CE: 12. Standard curves were generated for each substance of interest.

Molecular Biologic Methods

Routine biochemical variables in patient samples were measured by National Health Service hospital laboratories. NOx (NO2 and NO3) was measured by chemiluminescence with a Sievers Nitric Oxide Analyzer (GE Analytical Instruments, Manchester, UK) as per protocol. Protein concentrations were determined by Bradford assay and Nanodrop. Tissue genotyping was performed using ReadyMix Taq PCR Reaction Mix (Sigma-Aldrich). Tissue mRNA expression was determined by iScript cDNA reverse transcription kit, followed by quantitative RT-PCR (iQFast SYBR Greenmix with ROX (BioRad, Hertfordshire, UK) using a 7900HT Fast System, Applied Biosystems (Life Technologies, Paisley, UK). All reactions were performed in duplicate, and several “housekeeper” sequences were optimized for different types of biologic sample, with β2m the most stably expressed gene. Primer sequences are listed in the complete methods.

Proteins were separated and identified using Western blotting and SDS-PAGE according to standard protocols. A polyclonal goat DDAH1 antibody was produced by our own group. UMOD antibody was purchased from Sigma-Aldrich, and the LI-COR Odyssey system (Cambridge, UK) was used for protein detection and quantification. Mouse urine UMOD protein was quantified by ELISA according to manufacturers’ protocol (Uscn Life Science Inc., Hubei, China).

Histologic Methods

Frozen Sections

Kidneys from the ROSAYFP reporter mouse were fixed overnight in 4% paraformaldehyde, then 30% sucrose in PBS until full tissue submersion (about 4 hours). Specimens were embedded in optimal cutting temperature compound and snap frozen onto cork discs in methanol cooled on dry ice and stored at −80°C. Sections (5 μM) were cut at −20°C (cryotome CM 1850, Leica Microsystems, Wetzlar, Germany) onto poly-l-lysine–coated glass slides. Slides were studied using a Leica SP5 microscope.

Paraffin Sections

Harvested organs were fixed in 4% paraformaldehyde overnight and then stored in 70% ethanol. Tissues were embedded in paraffin blocks by an automated processor; 5-μM sections were cut on a microtome and attached to poly-l-lysine–coated glass slides before staining with periodic–acid Schiff or picrosirius red.

Collagen Content

Kidney sections were assessed for collagen deposition using a Manual Zeiss Axiohot microscope (Zeiss, Thornwood, NY) through circularly polarized light. Tissue images were obtained with a 2.5× objective lens, recorded on a digital camera (Canon EOS-40D digital SLR; Canon Inc., Tokyo, Japan) and viewed through Canon Utilities EOS software (Canon Inc.). Images were analyzed using a Fiji software macro (written by Dr. Dirk Dormann, head of microscopy, MRC Clinical Sciences Centre, Imperial College, London, UK) to quantify collagen in each whole kidney slice, thus obviating bias. Percentage collagen deposition per total section area (pixels) could then be reported.

Statistical Analyses

Statistical analyses were performed using the Prism software package (GraphPad Inc.). All data are presented as mean ± SEM. For comparisons between two groups for a single variable, a Mann–Whitney U test (nonparametric data or where a normal distribution could not be assumed) was used. Differences between multiple groups were assessed by one-way ANOVA, followed by Bonferroni post-test for multiple comparisons. Two-way ANOVA was used where comparisons were being made between groups subject to two experimental variables, followed by Bonferroni post-test for multiple comparisons. A P value <0.05 was considered to represent a statistically significant difference.

Proteomic Analysis (LC-MS/MS)

Urine samples were retrieved from −80°C and thawed in an Eppendorf Thermomixer Comfort mixer at 22°C, 900 g for 10 minutes, and then centrifuged at 5°C, 13,000 g for 3 minutes. Two hundred microliters of sample was mixed 1:5 with 0.1% trifluoroacetic (TFA) acid and subjected to two different solid phase extraction (SPE) steps; reverse-phase SPE (Waters Oasis HLB cartridges, Waters Corp., Milford, MA) and cation exchange SPE (Waters Oasis MCX cartridges). SPE was performed using a manual vacuum manifold (24-port vacuum manifold; Thermo Scientific) and LC-MS grade solvents (Fisher Scientific). Dried samples were solubilized in 40 μl of 0.1% TFA, sonicated for 10 minutes, and then centrifuged at 5°C, 13,000 g for 10 minutes. Ten microliters of supernatant was transferred to a Bioquote Ltd. 0.2-ml lubricated hydrophobic PCR tube and placed into an autosampler vial ready for LC-MS injection and analysis. A Dionex (Thermo Scientific) Ultimate 3000 nano-liquid chromatography system was used to separate peptides.
before mass spectrometric analysis. A volume of 4 μl was loaded onto a trap column (Acclaim Pepmap 100; Thermo Scientific) at 8 μl/min in 98% water, 2% acetonitrile, 0.1% TFA. Peptides were then eluted online to an analytical column (Acclaim Pepmap RSLC; Thermo Scientific) and separated using a ramped gradient with conditions: initial 5 minutes with 4% solvent B (96% solvent A), then 90 minute gradient 4%–55% solvent B, then 10 minutes isoetric at 100% solvent B, then 5 minutes isocratic at 4% solvent B (solvent A: 98% water, 2% acetonitrile, 0.1% formic acid; solvent B: 20% water, 80% acetonitrile, 0.1% formic acid). Eluted peptides were analyzed using LTQ XL Orbitrap (Thermo Fisher Scientific) operating in positive polarity, top four collision-induced dissociation (CID) method. Ions for dissociation were determined from initial 15,000 resolution MS scan (event 1) followed by CID on the top four most abundant ions. CID conditions were as follows: default charge state, 2; 2.0 m/z isolation width; normalized collision energy, 35.0; Activation Q value, 0.25; activation time, 30 ms; lock mass value, 445.120030 m/z.

Proteomic Data Analysis
Raw files were searched against UniprotKB/Swiss-Prot database (version 10032012) restricted to mouse entries using Mascot version 2.3.01. Relative quantification of peptides was performed by extracted ion chromatograms of parent ions using software developed in-house.

Human Cohort Data Methods
Participants
An appropriate research ethics committee, National Health Service management, and institutional review board approvals were in place for both cohort studies. Studies were conducted in accordance with the Declaration of Helsinki principles, and all participants provided written informed consent.

The London Protocol Biopsy Study consisted of patients undergoing protocol biopsy following first-kidney-alone transplant as per routine clinical care. Participants recruited from the Royal Free Hospital National Health Service Trust transplant clinic. The Virginia cohort underwent similar protocol biopsies except that two patients with kidney-after-liver transplant were also included. Follow-up biochemistry was performed in local laboratories as per routine clinical care; eGFR results were obtained with a minimum interval of 1 week between data points in the London study and monthly in the Virginia study.

Biopsy Tissue Quantitative RT-PCR
Protocol biopsy samples were preserved in RNA Later (Qiagen) at the bedside, before being stored at –70°C. Tissue was lysed in a 1% β-mercaptoethanol–containing proprietary lysis buffer (RILT, Qiagen). RNA was extracted using a column system (RNeasy kit, Qiagen) according to the manufacturer’s instructions. No carrier RNA was used. Resulting RNA samples were examined using a Nanodrop spectrophotometer (Thermo Scientific) before reverse transcription. First strand cDNA was synthesized using qScript cDNA Synthesis Kit (Quanta Biosciences, Gaithersburg, MD).

In the London cohort, primers were designed to amplify cDNA from human DDAH1. Primer sequences for human control genes were publicly available or listed elsewhere.51 Absolute quantification of cDNA was performed on a 7900HT Real-Time PCR System (Applied Biosystems, Life Technologies). Quantitative RT-PCR results from the genes of interest were normalized to the three most stable of six control genes: ACTB, β-2 microglobulin (B2M), hypoxanthine phosphoribosyltransferase 1 (HPRT), ribosomal protein 13A (RPL13A), succinate dehydrogenase complex subunit A flavoprotein (SDHA), and TATA box binding protein (TBP). In the Virginia cohort, a Taqman primer approach was used. Expression of DDAH1 and AGXT2 was performed and corrected to a single reference gene (B2M). In both cohorts, genes of interest were normalized to vary between zero and one. Ten samples were quantified at both sites, demonstrating high levels of intercenter agreement.

Uromodulin in the human cohort was measured using ELISA (MD Bioproducts, Zurich, Switzerland) as per manufacturer’s instructions.

Statistical Analyses
A multilevel multivariable approach was used to model eGFR over time in each of the two cohorts. Follow-up was commenced at the time of protocol biopsy, and GFR measurements (according to Modification of Diet in Renal Disease) out to 1 year after examination of biopsy specimens. The eGFR values were nested within patients using both a random intercept and random slope and the slope of eGFR quantified by the inclusion time variable. The effect of gene expression on eGFR decline was quantified by the addition of an expression×time interaction. Residuals were examined to ensure there was no substantial deviation from normality.

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DISCLOSURES
None.

REFERENCES


11. Wilcox CS: Role of macula densa NOS in tubuloglomerular feedback.


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