

Metabolomic Profiling of Arginine Metabolome Links Altered Methylation to Chronic Kidney Disease Accelerated Atherosclerosis

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Abstract

Atherosclerotic cardiovascular disease is the leading cause of death in patients with chronic kidney disease (CKD), but the mechanisms underlying vascular disease has not been fully understood. As the nitrogen donor in nitric oxide (NO[•]) synthesis, arginine and its metabolic products are integrally linked to vascular health and inflammation. We hypothesized that derangements in this pathway could explain, in part, increased atherosclerotic risk in CKD. We developed a targeted metabolomic platform to profile quantitatively arginine metabolites in plasma by liquid chromatography tandem mass spectrometry (LC/MS). Male low-density lipoprotein receptor deficient (LDLR^{-/-}) mice at age 6 weeks were subjected to sham or 5/6 nephrectomy surgery to induce CKD. Subsequently, the animals were maintained on high fat diet for 24 weeks. Targeted metabolomic analysis of arginine metabolites in plasma was performed by isotope dilution LC/MS including asymmetric dimethyl arginine (ADMA), symmetric dimethyl arginine (SDMA), N-mono-methylarginine (NMMA), arginine and citrulline. Although elevated plasma levels of ADMA and SDMA were found in the CKD mice, only higher ADMA level correlated with degree of atherosclerosis. No significant differences were noted in levels of NMMA between the groups. CKD mice had high levels of citrulline and arginine, but ADMA levels had no correlation with either of these metabolites. These findings strongly implicate altered arginine methylation and accumulation of ADMA, may in part contribute to CKD accelerated atherosclerosis. It raises the possibility that interrupting pathways that generate ADMA or enhance its metabolism may have therapeutic potential in mitigating atherosclerosis.

Keywords: Chronic kidney disease; Animal model; Arginine methylation; Atherosclerosis; Asymmetric dimethyl arginine

Abbreviations: ADMA: Asymmetric Dimethyl Arginine (20¹⁵dimethylarginine); BUN: Blood Urea Nitrogen; CKD: Chronic Kidney Disease; CAD: Coronary Artery Disease; CV: Cardiovascular; DDAH: Dimethyl Arginine Dimethyl Amino Hydrolase; eNOS: endothelial Nitric Oxide Synthetase; ESI: Electrospray Ionization; EIC: Extracted Ion Chromatogram; FIA: Flow Injection Analysis; HILIC: Hydrophilic Interaction Liquid Chromatography; LC/MS: Liquid Chromatography/Mass Spectrometry; 2015Low-density Lipoprotein Receptor Deficient; MRM: Multiple Reaction Monitoring; NMMA: 2⁰¹⁵-L-Arginine; NO: Nitric Oxide; PRMT: Protein Arginine Methyltransferases; SDMA: Symmetric Dimethyl Arginine (2⁰¹⁵dimethylarginine)

Introduction

Coronary artery disease (CAD) and cardiovascular (CV) events associates with chronic kidney disease (CKD) and is the leading cause of death in patients with CKD (>10-fold mortality). Indeed, CV events and mortality are more likely outcome than progression to end-stage renal disease (ESRD) in CKD 2015-sectional studies have demonstrated that the traditional risk factors are only partially predictive of CAD in CKD subjects, implying the presence of additional CKD- specific risk factors 2015In response to physiologic stimuli, endothelial cells dynamically regulate arterial vascular tone by producing vasodilators and vasoconstrictors. Risk factors for atherosclerosis, such as CKD, interfere with this response, promoting endothelial dysfunction and atherosclerosis. One key regulator is nitric oxide (NO), which is generated from L-arginine by endothelial nitric oxide synthase (eNOS) in the presence of cofactors such as tetrahydrobiopterin. Gaseous NO diffuses to vascular smooth muscle cells and activates guanylate cyclase, which in turn elevates cyclic guanosine monophosphate to promote vasodilation. NO's antithrombotic nature prevents platelet

aggregation, promotes fibrinolysis and decreases smooth muscle 2015

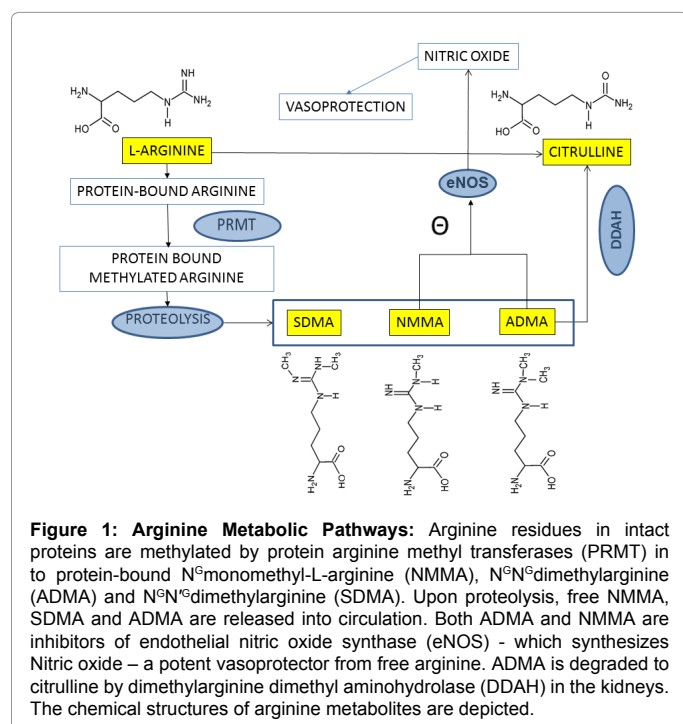
Asymmetric dimethylarginine (ADMA:20¹⁵) is the naturally occurring dimethylated modification of arginine and is a known inhibitor of NOS. One pathway for producing ADMA is proteolysis of methylated proteins which are formed by protein arginine methyl transferases (PRMT)2015Once released into plasma, ADMA can inhibit eNOS and decrease NO bioavailability, causing endothelial dysfunction. The enzyme dimethyl arginine dimethyl amino hydrolase (DDAH) metabolizes ADMA to generate dimethylamine and 2015DDAH has two isoforms of which DDAH 1 is thought be the primary enzyme responsible for ADMA degradation 2015the proximal tubules of kidneys can reabsorb almost all of the filtered L-arginine, very little ADMA is reabsorbed or excreted into urine. The majority of filtered ADMA is degraded into citrulline and dimethylamine by the renal DDAH as the kidneys has abundant amount of DDAH1. Thus in CKD, loss of DDAH1 activity may limit ADMA breakdown 2015Symmetric dimethyl arginine (SDMA; 2⁰¹⁵), a stereo isomer of ADMA is also produced by proteolysis following PRMT methylation of protein-bound arginine, but has no NOS inhibitory activity and is renally excreted.20¹⁵-L-arginine (NMMA) is the precursor of both ADMA and

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SDMA and is a more potent but less abundant NOS inhibitor. The effect of these methylation products on hypertension and cardiovascular morbidity in both CKD and healthy populations is well 2015Human studies have demonstrated that plasma ADMA levels are increased in CKD, hypertension, diabetes, obesity and metabolic 2015and are able to predict CV 2015Higher ADMA levels also predict cardiovascular events in dialysis patients 2015CAD related mortality in pre-dialysis CKD patients2015 and associates with CKD progression 2015ADMA is known to promote renal collagen and TGF2015production, thus promoting renal fibrosis and partly explaining the association with CKD progression 2015While these associative studies raise the possibility that altered arginine metabolism may contribute to atherosclerosis in CKD, a direct link between degree of atherosclerosis and the arginine metabolome has not been previously tested in experimental models of CKD atherosclerosis. In this study, we demonstrate altered arginine methylation is linked to degree of atherosclerosis in a CKD mouse model, raising the possibility of a therapeutic potential of interrupting this pathway in CKD-atherosclerosis.

Methods

Reagents and materials

Male C57BL/6 2015mice were purchased from Jackson Labs, Bar Harbor, ME. Authentic and isotopically labelled standards were purchased from the following vendors: NMMA, SDMA and 2₀15SDMA (Santa Cruz, CA2015ADMA (Novachem, Australia); creatinine, D₃ creatinine, arginine and citrulline (Sigma-Aldrich); ²⁰1₅Citrulline and ²⁰1₅Arginine (Cambridge Isotope Laboratories, MA). All LC reagents were purchased from Sigma Aldrich, St. Louis, MO. Rodent diets were purchased from 2015and high fat diet from Harlan Teklad.

Mouse models

All animal procedures were approved by the University of

Michigan Committee on Use and Care of Animals. Six week old male C57BL/6 2015mice were fed 2015standard rodent diet that has 200 ppm cholesterol, 28.5% protein, 13.5% fat and 58.0% carbohydrates by calories. Mice were housed in a climate-controlled, light-regulated facility with a 12:12 hour light-dark cycle and water ad libitum. At age 7 weeks, mice were subjected to either to sham surgery (Control, n=11) or to 5/6 nephrectomy to induce CKD (CKD, n =11). This was accomplished by removing entire right kidney in a first procedure and then subsequent removal of two thirds left kidney by dissection after one week interval. At 9 weeks of age, mice in each group were fed on high fat diet containing 19.5% protein, 40.5% fat, 0.5% cholesterol and 40.0% carbohydrates from Harlan Teklad (TD00243). Murine systolic blood pressure was measured by the IITC Life Science blood pressure system (Woodland Hills, CA) with a highly sensitive photoelectric sensor. The accuracy of measurements with five or more successful readings was obtained and secured by regular calibration of the pressure transducer. Blood was collected from saphenous veins in living mice with tubes containing dry ethylenediaminetetraacetate (EDTA). Hematocrit was measured by 2015Micro-Hematocrit centrifuge with Digital Hematocrit Reader (2015Company).

Analysis of kidney function

Plasma creatinine levels was measured by liquid chromatography electron spray ionization and tandem mass spectrometry (LC/ESI/MS) using an Agilent 6410 MS coupled with 1200 LC system (Agilent, New Castle, DE), as described previously 2015of plasma (n=11, each group) and 2015of deuterated creatinine (10 2015were added into 2015of 20 mM ammonium acetate solution, subjected to protein precipitation by 85% 2015of the supernatant was then injected for LC/MS analysis. A hydrophilic interaction chromatography (HILIC) was performed utilizing Luna Phenomenex column (2015, 2015Torrance, CA)) with an isocratic gradient of 85% acetonitrile with 20 mM ammonium acetate for 5 min at flow rate of 0.3 mL/min. The ion transition of 2015114 to 201544 for creatinine and 2015117 to 201547 2015creatinine was monitored in the multiple reaction monitoring (MRM) mode. The creatinine concentration in each plasma sample was determined by comparing the peak areas of the creatinine 2015-creatinine for the above transitions. Blood urea nitrogen (BUN) was measured directly on IDEXX VetTest 8008 chemistry analyzer (Westbrook, Maine) using dry slide technology.

Analysis of atherosclerosis

At 24 weeks mice were anesthetized, and the thoracic cavity was exposed and a small incision was made in the right cardiac auricle, and a cannula was inserted into the left ventricle. Through the left ventricle, the animal was perfused with phosphate-buffered saline until the eluent from the right auricle became clear, and then the left ventricle was injected with 3 ml of 10% buffered formalin. Finally, the entire mice were immersed in the fixative 2015. Each aortic tree was microdissected to remove adventitial fat and stained with Oil Red O (Sigma) to visualize neutral lipids, pinned on wax plates. The images of the aorta were captured on a digital camera. 2015quantification was performed with Image Pro software (Media Cybernetics, Bethesda, MD). The lesional areas are represented as ratios between surface area of atherosclerotic lesion stained with Oil Red O to the surface area of the entire aortic tree (n=11, each group).

Arginine metabolome profiling by LC/MS

The detailed method development and chromatography

2015depicts the extracted ion chromatogram (EIC) for the MRM transitions for ADMA (Panel A)2015ADMA(Panels B), SDMA (Panel C),2015(Panels D), NMMA (Panel E), arginine (Panel F), ²⁰1₅arginine (Panel G), citrulline (Panel H) and 2₀15(Panels I) with the optimized HILIC separation. The MRM transitions noted in 2015utilized for quantitative measurements of arginine metabolome in plasma.

CKD mouse model has biochemical evidence of CKD and increased atherosclerosis

The CKD mice at 24 weeks had significantly higher plasma creatinine (1.75 2015.34 mg/dL; n = 11; p<0.001) and BUN; (44.2015.17 mg/dL; n = 11; p<0.0001). The CKD mice had significantly lower body weight and hematocrit. The CKD mice did not show significant differences in cholesterol levels, mineral metabolism (Calcium, Phosphorus and intact parathyroid hormone) or blood 2015 We performed 2015analysis of the entire aorta and stained with Oil Red O to determine lesion area. Figure 4 Panel A and Panel B depict a representative control and CKD mouse aortic following Oil Red O stain. The CKD mice had increased atherosclerotic lesion area compared with control mice (0.201504 vs.0.2015.01; (n=9); p<0.05) (Figure 4: panel C; n=11 per group). The data strongly support induction of CKD as a major factor that accelerates atherosclerosis in this model.

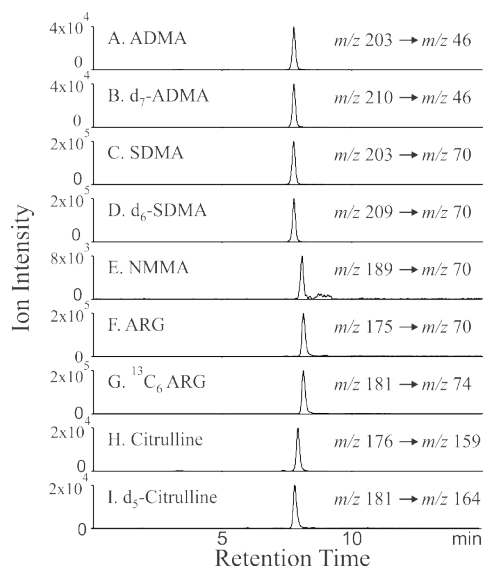


Figure 2: Detection of arginine metabolites by LC/MS: Extracted ion chromatograms of ADMA (A), D₇ ADMA(B), SDMA (C), D₆ SDMA (D), NMMA (E), arginine (F), ¹³C₆- arginine (G), citrulline (H) and ¹³C₅ citrulline (I) are shown. Hydrophilic interaction chromatography (HILIC) of authentic compounds and their internal standards was performed using Phenomenex HILIC column with solvent 10mM ammonium acetate and 100% acetonitrile with 0.1% formic acid. The column was equilibrated with 95% solvent B and 5% solvent A initially. The gradient was: 95-15% solvent B over 8 min, 15% solvent B for 6 min, 15-95% solvent B for 1 min and then finally 95% solvent B for 10 min. The elute was subjected electrospray ionization mass spectrometry (ESI/MS) and the analyte was detected in the multiple reaction monitoring mode (MRM). X axis is denotes time of elution during chromatographic run and the Y axis represents the ion intensity of the extracted ion chromatogram of the daughter ion following ESI of the analyte in the MRM mode. The MRM transitions that are followed for the individual analyte are depicted with each parent molecular ion to daughter fragment that was monitored.

Altered arginine methylation in CKD mice

Plasma ADMA, SDMA and NMMA levels were measured in plasma collected from the Control and CKD mice (n = 11 for each group).The plasma ADMA level was elevated in CKD mice compared to control mice (0.2015vs 0.20150.2015p<0.012015Panel A). Similarly plasma SDMA was elevated in CKD mice compared to control mice (0.201501 2015 vs.0.2015.01 μM; p<0.05; 2015The values for NMMA were not different between the two 2015Arginine methylation index, defined as the ratio of dimethylated arginines to the monomethylated precursor (ADMA+SDMA)/NMMA2015was significantly higher in the CKD mice, when compared to the control mice (29.20152.71 vs. 20.2015.31 p<0.05; 2015Figure 3 Panels E and F depict elevations of plasma arginine (80.66 2015vs. 6220154.2015p<0.05) and Citrulline ((15.07 2015vs. 5.79 2015p<0.05) in CKD mice compared to control mice. These data strongly suggest that CKD alters arginine metabolism, raising the possibility that such alterations may diminish NO bioavailability, contributing to vascular dysfunction.

Levels of altered arginine methylation products and their substrates correlate with each other

The ADMA levels of both control and CKD mice together correlate with SDMA levels (r = 0.549, p < 0.01) while both arginine and citrulline levels correlate with each other (r = 0.449, p < 0.05; 2015using pearson correlation. The substrate for ADMA production-arginine and the by-product of ADMA degradation -citrulline levels do not correlate with ADMA levels (arginine r=0.36 p=0.09; citrulline r=0.24; p=0.582015This implies that plasma citrulline is predominantly derived from arginine and not from ADMA. We also performed Spearman correlation analysis between ADMA, SDMA, NMMA and arginine and between citrulline and arginine which showed similar results (Data not shown).

ADMA levels, but not other arginine metabolites, are strongly associated with atherosclerotic burden

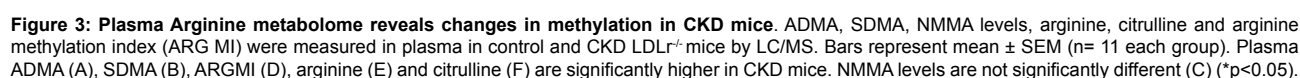
We tested whether arginine metabolites correlated with atherosclerotic burden by comparing levels of the metabolites with lesional area, a measure of degree of atheroma in control and CKD mice by performing Pearson Correlation 2015). Only ADMA levels correlated with the lesional area (r = 0.64, p<0.01) while SDMA levels did not (r = 0.02, p>0.05). Similarly, NMMA and arginine methylation index did not correlate with degree of atherosclerosis (Data not shown). The association of ADMA to atherosclerosis was stronger in the CKD mice. This data strongly supports the notion that ADMA might directly contribute to degree of atherosclerosis, perhaps by inhibiting eNOS and contributing to decreased NO production.

Discussion

In this study, we utilized a mouse model of atherosclerosis to

	CONTROL (n=11) Mean ± SD	CKD (n=11) Mean ± SD	p value
Body weight (g)	33.52 ± 1.5	27.39 ± 0.6	<0.01
Blood Pressure (mm Hg)	102.70 ± 4.1	116.30 ± 9.2	NS
Hematocrit (%)	61.0 ± 0.9	52.91 ± 1.2	<0.0001
Serum Creatinine (mg/dL)	0.97 ± 0.34	1.75 ± 0.54	<0.001
Blood urea nitrogen (mg/dL)	28.25 ± 1.17	44.17 ± 1.79	<0.0001

Table 2: Physiological and Biochemical characteristics of study animals (n=11; each group)

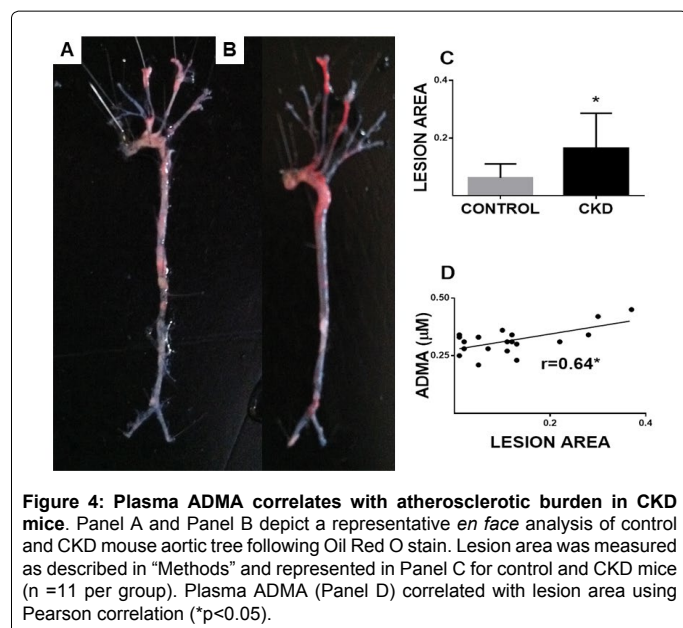


*. Correlation is significant at the 0.05 level (2-tailed).

address whether alterations in arginine metabolism, in part accounts for increased atherosclerosis observed in CKD. We utilized LC/MS to profile and quantitatively measure arginine and its methylated derivatives in plasma. We established that ADMA, SDMA, NMMA levels and the arginine methylation index are elevated in CKD mice. ADMA levels are directly related to the extent of atherosclerosis in CKD mice, suggesting a central role for altered NO bioavailability in atheroma formation in this model. Finally, we provide evidence that the ADMA elevation is not entirely related to availability of its substrate arginine or correlated to levels of its byproduct citrulline but might be a result of potential enzymatic pathways that lead to formation of methylated proteins or its degradation.

determine the instrument response and to account for ion suppression and matrix effects for each specific compound in contrast to previously published methods that use one labeled standard for many. This method is the first to use labeled SDMA to accurately determine its concentration independent of ADMA. Using the appropriate isotope labeled standard, this methodology enables a very accurate measurement of the arginine metabolites with minimal interassay and intraassay variability. This method also eliminates derivatization, solid phase extraction and other complicated procedures like ultrafiltration and uses a straight forward protein precipitation process, minimizing sample loss and expense.

The LDL receptor deficient mouse is a well-characterized model for atherosclerosis that develops extensive lesions in aortic root and branches and perivascular system as described 2015. The effect of CKD in this mouse model has also been described previously and mainly results in acceleration of the lesions similar to non-CKD 2015. The mouse however does not develop coronary atherosclerosis and hence we measured aortic root lesion area as this is the most reproducible measurement. Following 5/6 nephrectomy, the mice demonstrate features of CKD like increased BUN and creatinine. The mice do not have increased blood pressure or changes in the calcium phosphorus metabolism, making CKD the sole variable that could potentially



contribute to atherosclerosis. The atherosclerosis in this model is accelerated with high fat diet and similar models in the 2015are previously 2015

Interestingly, a study by Jacobi et al reported no change in ADMA levels in subtotal nephrectomized mice in 2015background fed on chow diet for 12-2015These findings are in contrast to earlier publications that show increased ADMA levels in CKD models and our current 2015Also surprisingly in that work, 2015CKD mice did not have higher atherosclerotic burden when compared with DDAH 1 overexpressing mice on an 2015background. Over expression of DDAH1 in this mice model however predictably decreased ADMA levels but did not change atherosclerotic lesions. It is likely that these differences are attributable to strain differences, more modest reduction in renal function and diet compared to our study. In our work, CKD mice have accelerated atherosclerosis, manifested as increased luminal lipid accumulation, elevated aortic plaque, necrotic core, fibrosis as well as greater luminal narrowing. Selective alterations of arginine methylation were identified in our study and may in part account for propensity towards increased atherosclerosis in this model. Indeed, the atherosclerotic burden correlated only with ADMA levels, but not SDMA, NMMA or arginine methylation index.

CKD is associated with decreased NO bioavailability either due to decrease in production due to substrate (arginine) limitation, increased levels of ADMA or increased utilization or presence of increased 2015We demonstrate in our work that changes in ADMA in our model is not related to free arginine and citrulline in plasma suggesting that ADMA levels are not entirely just a consequence of increased arginine levels due to decreased renal function. Elevated ADMA levels are probably a result of increased flux in this pathway, partly due to increased methylation of proteins by PRMT, decreased renal excretion and decreased DDAH1 activity. In a previous study, it was demonstrated that ADMA levels are elevated in puromycin-induced CKD rats due to reduced DDAH 2015Studies in mongrel dogs have also revealed microvascular endothelial changes in CKD dogs that was associated with increased ADMA levels and down regulation of DDAH-II 2015changes in DDAH might be associated with loss-of-function polymorphisms of a2015gene, functional inhibition of the enzyme by

oxidative stress in CKD and end-stage renal disease, or 2015DDAH1 overexpression in the 2015in a previous study demonstrated decreased plaque area in a non CKD model associated with lower ADMA 2015but this has not been tested in CKD mice. Future studies will need to focus on DDAH isoforms and the effect of DDAH overexpression in relevant CKD models.

In clinical studies higher ADMA levels predicted cardiovascular events when compared to control patients in pre-dialysis subjects 2015In a large clinical study, Wang et al demonstrated that arginine methylation index correlated with CVD 2015Therefore, we tested the utility of this measurement in our CKD mouse model, but this measure did not correlate with atherosclerotic burden, perhaps due to small numbers in our study. ADMA together with related markers of oxidative stress like myeloperoxidase could potentiate development of 2015Renal cyclooxygenase 2 (COX-2) inhibition raises ADMA levels and could explain the increased cardiovascular morbidity of COX-2 inhibitors adding to ADMA's role in cardiovascular 2015Thus, ADMA together with inflammatory and oxidative markers could play a central role in the accelerated atherosclerotic burden in CKD.

Our model has several strengths. We demonstrate for the first time that in a pathophysiologically relevant model of CKD atherosclerosis altered arginine methylation and a high ADMA levels correlate with atherosclerotic burden. These changes occur even with modest CKD suggesting that these pathways could be relevant in early CKD. The mouse model does not have common associated features of CKD such as hypertension, and abnormal mineral metabolism which make it an ideal model to study effect of mild CKD alone. The limitations of this work include small numbers in this study and lack of manipulations to alter ADMA levels to show causality with atherosclerotic burden. Manipulation of PRMT, DDAH or the alternate enzyme alanine-glyoxalate aminotransferase-2 (AGXT22015could provide such direct evidence and future studies need to focus on this issue. Finally, our findings raise the possibility that interrupting arginine methylation pathways could provide a therapeutic avenue for combating CKD-atherosclerosis.

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