Myeloperoxidase derived oxidants damage artery wall proteins in an animal model of chronic kidney disease accelerated atherosclerosis

Lixia Zeng1, Anna V. Mathew1, Jaeman Byun1, Kevin B. Atkins1, Frank C. Brosius III1,2, and Subramaniam Pennathur1,2*

From the 1 Department of Medicine, Division of Nephrology, 2 Molecular and Integrative Physiology, University of Michigan, Ann Arbor MI 48105

Running title: Myeloperoxidase oxidizes artery proteins in kidney disease

*AVM and LZ contributed equally to this work

*To whom correspondence should be addressed: Subramaniam Pennathur: Division of Nephrology, University of Michigan, 5309 Brehm Center, 1000 Wall Street, Ann Arbor MI 48105; spennath@umich.edu.

Keywords: chronic kidney disease, atherosclerosis, myeloperoxidase, macrophages, oxidative stress, oxidized amino acids, 3-chlorotyrosine, 3-nitrotyrosine, mass spectrometry

ABSTRACT

Increased myeloperoxidase (MPO) levels and activity are associated with increased cardiovascular risk among individuals with chronic kidney disease (CKD). However, a lack of good animal models for examining the presence and catalytic activity of MPO in vascular lesions has impeded mechanistic studies into CKD-associated cardiovascular diseases. Here, we show for the first time that exaggerated atherosclerosis in a pathophysiologically relevant CKD mouse model is associated with increased macrophage-derived MPO activity. Male 7-week-old LDL receptor–deficient mice underwent sham (control mice) or 5/6 nephrectomy and were fed either a low-fat or high-fat, high-cholesterol diet for 24 weeks, and the extents of atherosclerosis and vascular reactivity were assessed. MPO expression and oxidation products, and the protein-bound oxidized tyrosine moieties 3-chlorotyrosine, 3-nitrotyrosine, and o,o′-dityrosine were examined with immunoassays and confirmed with mass spectrometry (MS). As anticipated, the CKD mice had significantly higher plasma creatinine, urea nitrogen, and intact parathyroid hormone along with lower hematocrit and body weight. On both the diet regimens, CKD mice did not have hypertension but had lower cholesterol and triglyceride levels than the control mice. Despite the lower cholesterol levels, CKD mice had increased aortic plaque areas, fibrosis, and luminal narrowing. They also exhibited increased MPO expression and activity (i.e., increased oxidized tyrosines) that co-localized with infiltrating lesional macrophages and diminished vascular reactivity. In summary, unlike non-CKD mouse models of atherosclerosis, CKD mice exhibit increased MPO expression and catalytic activity in atherosclerotic lesions, which co-localize with lesional macrophages. These results implicate macrophage-oxidized MPO in CKD-accelerated atherosclerosis.

Chronic kidney disease (CKD) affects 15% of Americans, and cardiovascular disease (CVD) continues to be the leading cause of mortality in...
the specific role of MPO in CKD-accelerated atherosclerosis remains only partly understood. In general, human studies in patients with CKD have suggested that MPO plays an important role. For example, MPO-derived protein carbamylation is associated with CVD in the CKD population (46-49). Plasma MPO levels increase with each stage of CKD and are linked to CVD mortality in patients with end-stage renal disease (ESRD) who are undergoing peritoneal dialysis (50-53). In a recent study, our group reported that both MPO and 3-chlorotyrosine levels rise in the presence of CAD at various stages of CKD (54). It is important to note, however, that most of these studies are associative; hence, causality remains unclear.

Lack of good animal models to study the role of MPO in CKD-associated atherosclerosis renders the determination of causality challenging. Despite strong evidence of MPO activity in human atherosclerotic lesions (28,38,55), mouse models of non-CKD atherosclerosis have undetectable MPO oxidation products in mouse atheroma (56). Paradoxically, MPO-deficient mice exhibit
exaggerated atherosclerosis, although a study overexpressing MPO human transgene in macrophages showed increased atherosclerosis (57). This lack of MPO activity in mouse atheroma of non-CKD mice further highlights the need for an appropriate CKD-atherosclerosis model that demonstrates MPO activity in atheromatous lesions and lends itself to the systematic study of MPO action in CKD. In this study, using a pathophysiologically relevant 5/6 nephrectomy model of CKD in the LDL receptor knockout (LDLr<sup>−/−</sup>) mouse, we tested the presence of MPO and its activity in aortic tissue. This model has been extensively studied and reported by our group and others as a model that exhibits accelerated atherosclerosis with CKD (58-61). These CKD mice, exhibit all the biochemical features of CKD, and demonstrate accelerated atherosclerosis without elevated blood pressure, diabetes, or worsened hyperlipidemia compared to non-CKD mice. Thus, the CKD LDLr<sup>−/−</sup> model is an excellent animal model for studying effects of CKD on atherosclerosis without the concomitant added risk factors. In contrast to non-CKD models, we report robust MPO expression and catalytic activity as demonstrated by elevated MPO oxidation products, that co-localize with macrophages in atherosclerotic lesions. This implicates macrophage derived MPO as a source of oxidants in the artery wall which might propagate CKD-accelerated atherosclerosis.

**Results**

**CKD LDLr<sup>−/−</sup> mice exhibit biochemical features of moderate CKD**

C57BL/6 LDLr<sup>−/−</sup> mice were subjected either to sham operation (CTL, n=20) or to 5/6 nephrectomy (CKD, n=21). At 9 weeks of age, the mice in each group were further randomly divided into two subgroups and fed on low fat diet (LFD) or high fat-high cholesterol diet (HFD). The mice in each group, CTL-LFD, CTL-HFD, CKD-LFD, or CKD-HFD, were maintained on these diets for either 12 or 24 weeks.

As shown in **Table 1**, serum blood urea nitrogen (BUN) and creatinine from CKD-LFD and CKD-HFD mice were elevated compared to their CTL counterparts, confirming the renal insufficiency of CKD mice. Serum BUN was 1.5 times, and serum creatinine was approximately 3 times higher in the CKD counterparts compared to their CTL counterparts indicating moderate CKD. There was a consistent decrease in renal function over time in all four groups that did not differ. Hematocrit in CKD mice on either diet was decreased at 12 weeks (p<0.05), and decreased further at 24 weeks (p<0.05). Despite the increased intact parathyroid hormone (iPTH), there was no change in the serum calcium or phosphorus levels. Unlike human CKD, the CKD mice tended to have lower blood pressure compared to controls over time, though this trend did not reach statistical significance. There were no differences in the hemoglobin A1c (HbA1c) levels among all 4 groups.

Mice from all four groups gained significant body weight over time (p<0.001). Compared to the CTL mice, the CKD mice gained less weight at 12 weeks, and this difference persisted at 24 weeks. Although there was no significant difference at 12 weeks, the 24 week HFD fed mice in the CKD groups gained much more than their LFD mice (p<0.01).

**Effects of CKD and HFD on plasma lipid levels and lipoprotein profiles**

With the exception of triglycerides in CKD-LFD mice, plasma cholesterol and triglyceride concentrations were increased at 24 weeks in all diet categories, especially with HFD (p<0.0001; Table I). CKD mice fed HFD had decreased levels of cholesterol and triglycerides when compared to CTL mice fed HFD at 24 weeks (p<0.05). As expected, the HFD mice in each renal function category had higher cholesterol and triglycerides compared to LFD mice with similar renal function.

Lipid distribution among the various lipoprotein fractions was analyzed by fast protein liquid chromatography (FPLC) in pooled plasma samples from each group at 24 weeks. As shown in **Figure 1A**, mice on LFD had lower cholesterol in the very low density (VLDL) and low density lipoprotein (LDL) fractions than mice on the HFD. The CKD mice had considerably lower VLDL and LDL cholesterol, with unchanged high density lipoprotein (HDL) cholesterol in comparison with the CTL mice. Similarly, as shown in **Figure 1B**, CKD mice, in comparison to CTL mice in the same diet category had lower triglyceride content in the VLDL and LDL fractions. Mice on HFD had higher
triglyceride content in the VLDL and LDL fractions compared to mice on LFD. Therefore, overall CKD mice had a more favorable atherogenic lipid profile compared to CTL mice.

**Effects of CKD and HFD on aortic atherosclerotic lesions**

*En face* analysis of aortic tree lesion area and cross-sectional analysis at the root of the aorta were performed to assess atherosclerosis. As shown in Figure 2, Oil red staining of aorta tree and subsequent comparative morphometry revealed that all groups demonstrated increasing atherosclerotic lesions with age. At 12 weeks, HFD diet accelerated atherosclerosis in both CTL and CKD mice. At 24 weeks, CKD HFD mice had greater atherosclerosis than CKD-LFD and CTL-HFD (p value<0.05) mice. The CKD-HFD mice had more advanced atherosclerotic lesions in aortic root (Figure 3), aortic arch, brachiocephalic arteries, thoracic aorta and abdominal bifurcations as shown in cross sections of aortic root stained with hematoxylin and eosin (H&E) (Figure 3A and 3B) and Masson Trichrome (Figure 3C and 3D). Specifically, at 24 weeks CKD-HFD mice (Figure 3B and 3D) had significantly elevated aortic plaque area fraction, luminal narrowing, and cellular filtration with significant fibrosis when compared to CTL-HFD mice (Figure 3A and 3C).

**Effects of CKD and HFD on the vasodilatory response**

Given the intimate linkage between atherosclerosis and vascular function, we assessed the cholinergic responsiveness of mouse aortic rings from the four mouse groups. CKD-HFD mice differed significantly in response to acetylcholine from that observed in rings from mice of the other three groups. The rings of CKD-HFD mice initially relaxed with acetylcholine. However, they then recontracted moderately and maintained a similar tension at higher concentrations of acetylcholine. This phenomenon is illustrated by the data points (3x10^{-3}M and 10^{-6}M acetylcholine) that lie above the interpolated relaxation curve for the CKD-HFD mice (Figure 4). The maximum vasodilatory response (Emax) for the aortic rings of CKD-HFD mice was significantly different compared to those observed for the other three groups (p<0.01 vs. CKD-LFD/CTL-HFD; p<0.0001 vs. CTL-LFD). There were no differences in the Emax to acetylcholine exhibited between rings from the CTL-LFD, CKD-LFD, or CTL-HFD mice (5.804±3.296%, 13.39±2.941%, 13.01±2.295%, respectively; Figure 4). There was no difference in relaxation in nitricergic (response to sodium nitroprusside) in any of the groups (not shown).

**Immunohistochemical staining and mass spectrometry assessment of MPO and tyrosine oxidation products in atherosclerotic lesions**

MPO can oxidize tyrosine moieties in proteins to generate oxidized tyrosines. While 3-chlorotyrosine is a specific marker for MPO, it can also form 3-nitrotyrosine and o, o′-dityrosine. We examined MPO expression and presence of oxidized tyrosines with immunohistochemistry. Figure 5 shows representative immunostaining of atherosclerotic lesions in aortic cross sections from LDLr⁻/⁻ mice at 24 weeks. MPO expression was observed in atherosclerotic lesions in CKD-HFD mice (Figure 5B) when compared to CTL-HFD mice (Figure 5A). Similarly, immunohistochemical staining for macrophage marker F4/80 protein and MPO-derived tyrosine modifications using specific antibodies for 3-chlorotyrosine, 3-nitrotyrosine, and o, o′-dityrosine demonstrated robust staining in the CKD-HFD mice at 24 weeks in comparison with the CTL HFD (data not shown).

In order to confirm activity of MPO in these lesions, we utilized stable-isotope-dilution mass spectrometry (MS), a highly sensitive and specific method to quantify the levels of protein tyrosine oxidative modifications: 3-chlorotyrosine, 3-nitrotyrosine, and o, o′- dityrosine in the aortic lesions. In contrast to immunooassays which are qualitative, MS provides accurate quantitative data which is essential to ascribe enzyme activity. All results were normalized for the precursor amino acid tyrosine. As shown in Figure 5C, at 24 weeks the average 3-chlorotyrosine levels (the specific MPO product) were markedly increased (156.3 ± 28.1 vs. 102.5 ± 19.6; p = 0.012) in CKD versus CTL mice fed HFD in the aortic tissue. Similarly, CKD LFD mice showed increased lesional 3-chlorotyrosine compared to CTL-LFD mice (94.1±50.9 vs. 2.6±1.7; p<0.0001). Hence CKD status, independent of the type of diet, augmented the presence of the MPO product 3-chlorotyrosine in atherosclerotic lesions.
The average 3-nitrotyrosine levels approximately doubled (2757±376.1 vs.1365±693.8, p = 0.01) in CKD compared to control aortic tissue. 3- nitrotyrosine was higher in CKD-LFD compared to CTL-LFD (1204±1133 vs. 137.3±65.3; p<0.05, Figure 5D).

\( o,o' \)-dityrosine levels were 1.36 fold higher in CKD-LFD compared to CTL-LFD mice (95.67±11.7 vs. 70.2±15.5; p<0.05), while the levels were unchanged between CKD-HFD mice and CTL-HFD mice (148.4±39.9 vs. 151.6±16.8; p = 0.70). \( o, o' \)-dityrosine in lesions of CKD-HFD were increased compared to the CKD-LFD mice (n=8, p<0.01, Figure 5E). These data suggest that tyrosine oxidation products are increased in aortic tissues of CKD mice and accentuated with HFD.

In addition to their increased presence, these oxidation products correlate with each other. Importantly, 3-chlorotyrosine a specific product of MPO correlated strikingly with 3-nitrotyrosine (\( r=0.93; \) p value <0.0001) suggesting MPO is the source of both of these products. The other correlations were less striking (3-chlorotyrosine versus \( o, o' \)-dityrosine \( r=0.50; \) p value=0.005; \( o, o' \)-dityrosine versus 3-nitrotyrosine \( r=0.64; \) p value=0.0001) suggesting that in addition to MPO other pathways might contribute to their formation (Figure 5F to 5H).

**MPO expression and its tyrosine oxidation products in atherosclerotic lesions in CKD-HFD mice**

Using a double labeling immunofluorescence technique, we found that Mac-2 (a macrophage marker), MPO, \( o,o' \)-dityrosine, 3-nitrotyrosine and 3-chlorotyrosine co-localized intensely in atherosclerotic plaques in the CKD-HFD mice. The labeling was concentrated at the luminal surface of the plaque areas (Figure 61 to L).

In order to ascertain the specificity and cross-reactivity of these antibodies, we generated oxidized bovine serum albumin (BSA) *in vitro* with conditions favoring chlorination (MPO-peroxide-chloride with resultant increase in 3-chlorotyrosine), nitration (MPO-nitrite-peroxide system with increase in 3-nitrotyrosine), and hydroxyl radical formation (copper-peroxide; with \( o,o' \)-dityrosine, *ortho-*tyrosine (*o*-tyrosine) and *meta-*tyrosine (*m*-tyrosine) generation) as described previously and elaborated in Supplemental Methods (55,62-64). The formation of the anticipated oxidized amino acids following the reactions was confirmed by MS. We then pooled the reaction mixtures to form a mixture of BSA containing all oxidized tyrosine modifications (*o*,*o'*-dityrosine, *o*-tyrosine, *m*-tyrosine, 3-nitrotyrosine, and 3-chlorotyrosine). We subsequently subjected the mixture to immunoprecipitation with the anti-3-chlorotyrosine, anti-3-nitrotyrosine and anti-*o*,*o'*-dityrosine antibodies. The pre and post-immunoprecipitation mixtures were tested for enrichment of modified tyrosines by MS following acid hydrolysis. Immunoprecipitation with anti-3-nitrotyrosine antibody showed ~ 3-fold enrichment of 3-nitrotyrosine but not of the other tyrosine moieties. Immunoprecipitation with anti-*o*,*o'*-dityrosine antibody resulted in ~18-fold enrichment of *o*,*o'*-dityrosine compared to pre-immunoprecipitation levels but not of the other modified tyrosines, confirming the specificity of these 2 antibodies (Table S1). In contrast, anti-3-chlorotyrosine antibody (utilizing the only commercially available antibody) did not result in enrichment of 3-chlorotyrosine (nor any other oxidized amino acid; Table S1). This could be potentially because a) the anti-3-chlorotyrosine antibody is not specific; b) is not sensitive as 3-chlorotyrosine generation was lower than the other modifications, or c) the antibody is more conducive to immunochemistry studies but may not work well in the immunoprecipitation reactions.

These results suggest that MPO derived from lesional macrophages co-localizes with 3-nitrotyrosine and *o*,*o'*-dityrosine residues in atherosclerotic plaques of CKD-HFD mice. However, given the non-specificity of the 3-chlorotyrosine antibody, we cannot ascertain the same for 3-chlorotyrosine, the specific marker for MPO. Taken in conjunction with the quantitative MS data which shows a strong correlation of 3-chlorotyrosine with other oxidative tyrosine moieties, the data is highly suggestive that MPO derived from lesional macrophages as the source of all the three oxidatively modified tyrosines in atherosclerotic lesions.

**Discussion**

MPO-mediated oxidative stress is associated with elevated CVD risk in CKD patients (65,66); however, testing whether MPO is pathogenically important in atherosclerosis in CKD
patients is difficult given a lack of strong animal models of atherosclerosis that also demonstrate significant MPO activity in atherosclerotic lesions. Our CKD atherosclerosis mouse model is unique and is the first to demonstrate increased MPO expression and activity in atherosclerotic lesions. The atherosclerotic lesions in this model clearly exhibit elevated 3-chlorotyrosine, a specific marker for MPO activity, in addition to 3-nitrotyrosine and \( o, \ o' \text{dityrosine} \) (two modified tyrosines that can be derived from MPO or alternate sources). Importantly, the oxidative tyrosine modifications show high-degree of correlation amongst each other, supporting the notion that MPO mediates all three oxidative modifications in this model. Furthermore, these oxidative MPO products co-localize with macrophages in the lesions, confirming the presence of catalytically active macrophage derived MPO in the lesions in this model.

The mouse model of atherosclerosis and CKD bears some similarities to human CKD and atherosclerosis, including elevated LDL cholesterol and biochemical features of moderate CKD (e.g., tripling of serum creatinine, mild anemia, and elevated iPTH levels with normal calcium and phosphorus levels consistent with mild secondary hyperparathyroidism). These mice do not develop diabetes and hypertension even when exposed to HFD, making this an ideal model for the study of cardiovascular complications of moderate CKD alone. The CKD mice also exhibit a more favorable lipid profile compared to the CTL mice when exposed to an HFD; however, they develop enhanced atherosclerosis, suggesting that CKD significantly accelerates atherosclerosis in this model beyond the influence of hyperlipidemia. To our knowledge, this is the first mouse model that demonstrates increased lesional MPO expression and catalytic activity likely derived from macrophages.

Macrophages are closely associated with atherogenesis and represent a major source of oxidative stress in CKD (51,67). However, the role of macrophage derived MPO in CKD associated atherosclerosis is yet to be explored using mechanistic models, as non-CKD animal models of atherosclerosis that demonstrate clear MPO involvement are not established. LDLr\(^{-/-}\) mice of C57BL/6 background, similar to our model on a high-fat and high-cholesterol diet, did not demonstrate remarkable MPO presence or activity in atherosclerotic lesions. This finding is dissimilar from human lesions, which have increased 3-chlorotyrosine, a specific product of MPO activity (56). This calls into question the appropriateness of mouse models for studying MPO-related atherosclerosis, as there appear to be notable differences between murine and human atherosclerosis. Moreover, MPO-deficient mice with both macrophage-specific and whole-body knockouts paradoxically demonstrated a 50% increase in atherosclerosis after 14 weeks of a high-fat, high-cholesterol diet, suggesting that alternate pathways are involved in the process. This inability to replicate the MPO activity evident in human atherosclerosis in mouse models has deterred further mechanistic studies. Thus, our CKD animal model that demonstrates robust MPO involvement in vascular lesions after 24 weeks of a high-fat, high-cholesterol diet is invaluable in developing a better understanding of atherosclerosis in CKD.

MPO expression is restricted to hematopoietic cells and is controlled by promoter, enhancer, and repressor elements in addition to transcription and growth factors that influence these elements (68). -463G/A polymorphism at one of the upstream Alu MPO promoter elements has been linked to increased incidence of coronary artery disease in the general population (69,70) and CVD incidence in CKD patients (71) and ESRD patients (72). This primate-specific promoter contains binding site for nuclear transcription factors, namely SP1-thyroid hormone-retinoic acid response element (73), peroxisome proliferator activated receptor alpha and gamma, retinoid X receptors (74), statins (75), and the liver X receptor (76), which promote MPO expression and is competitively inhibited by the estrogen (68). This promotor is absent in mice, a finding that may partially explain the marked absence of MPO or MPO activity in the mouse model and the failure of MPO-deficient mice to ameliorate atherosclerosis. Repopulating the bone marrow of LDLr\(^{-/-}\) mice with bone marrow from transgenic mice expressing human myeloperoxidase resulted in increased atherosclerosis (57). Another study, utilizing the overexpression of different human MPO allele polymorphisms in the promoter region, likewise demonstrated increased aortic lesions in male mice only, thus highlighting the role of estrogen in ameliorating MPO expression (77). Yet MPO
polymorphism and its effect on MPO expression are much more complicated (78); while some studies implicate the GG genotype with an increased risk of CVD (72,78), other studies have not clearly replicated this finding (79,80). Our model of CKD atherosclerosis is the first of its kind to demonstrate marked upregulation of mouse MPO and its specific oxidation products in response to reduced renal function and HFD in murine atherosclerosis. Similar to our work, apolipoprotein E deficient (apoE−/−) mice with chronic renal failure demonstrate enhanced atherosclerosis with increased macrophage infiltration and 3-nitrotyrosine expression in their atherosclerotic lesions, suggesting a similar process is plausible (81). However, this study did not specifically investigate if MPO or MPO-specific oxidation products are elevated. It is unclear how reduced renal function influenced MPO expression in these mice who have such low baseline MPO expression with functioning kidneys. One possible reason is the prolonged exposure to HFD for 24 weeks in our model, in addition to other factors altered by decreased renal function. Our demonstration using the quantitative measurement of MPO oxidation products co-localizing with macrophage markers suggests that macrophage-derived MPO may propagate enhanced atherosclerosis in this model. The correlation between these oxidized moieties strongly implicates MPO as the source of all of these modifications.

MPO scavenges the reactive nitrogen species generated in the vascular wall to modulate vascular reactivity by consuming vasodilator nitric oxide. Nitric oxide can also combine with NADPH oxidase-generated superoxide (O2−) to produce the reactive nitrogen species peroxynitrite (ONOO−), which has a very short half-life and causes nitration of proteins. In addition to decreased availability, nitric oxide production by itself could be the issue in this model as suggested by our earlier work demonstrating high levels of circulating methylated arginines that inhibit NO synthase (58). Thus, extensive atherosclerotic burden, modulation from the reactive nitrogen species, elevated methylated arginines, and nitric oxide consumption contribute to endothelial dysfunction that is associated with an increased risk of atherosclerosis disease in CKD (82). We report that this dysfunction is manifested as decreased vasodilation in response to cholinergic stimulation in CKD-HFD mice only. This is an interesting observation, as it clearly demonstrates the important interaction between diet and renal function necessary to induce endothelial dysfunction. Prior reports of endothelial dysfunction are limited to the double knockout of LDLr−/− with apoE−/− mice (83,84). These mice, whether on HFD or not, fail to exhibit reduced cholinergic vasodilation (85-87). These results are consistent with our findings for CTL-LFD and CTL-HFD mice. What is more, while there are reports of 5/6 nephrectomy inducing a reduction in cholinergic responsiveness, such research seems to have been conducted exclusively in rats (88,89). In these cases, the reduction in response was demonstrated with renal insufficiency but was associated with related hypertension and disruption in the angiotensin-dependent pathway. Clearly, our results show that reduction in cholinergic response is not dependent on kidney function alone but is rather a response to extensive atherosclerosis caused by the combination of decreased renal function and HFD in the absence of hypertension. Thus, our findings indicate that our model provides a foundation from which to explore the interaction of diet and renal function without the confounding influence of hypertension.

Our 5/6 nephrectomized LDLr−/− model achieved by surgical means, in contrast to uninephrectomy and 5/6 nephrectomy achieved via renal ablation, demonstrates sufficient and consistent loss of renal function to mimic moderate CKD as confirmed by creatinine and BUN measurements. As with humans, these CKD mice develop anemia and iPTH elevation, which confirms secondary hyperparathyroidism and effects consistent with an advanced degree of renal function. While both LDLr−/− and apoE−/− mice are well established atherosclerotic models, LDLr−/− mice exhibit more modestly elevated LDL cholesterol levels compared to apoE−/− mice. Our study demonstrates the development of atherosclerosis with HFD exposure in LDLr−/− mice at 12 weeks that becomes more pronounced at 24 weeks. This elevated atherosclerotic plaque burden was observed despite reduced plasma cholesterol and triglycerides compared with controls on the same diet. The LDLr−/− CKD mice also show lower triglyceride and cholesterol levels in the VLDL and LDL fractions compared to controls irrespective of diet. These findings differ from the prior literature on other uremic atherosclerosis models that use...
both LDLr−/− and apoE−/− mice with an exaggerated aortic lesional area independent of hypertension but, unlike our observations, exhibit marked hypercholesterolemia compared to controls on the same diet (60,90-94). In a study by Bro et al., uremic apoE−/− mice that were fed a regular diet for 22 weeks had 50% higher total plasma cholesterol concentrations than normal apoE−/− mice (94). Plasma triglyceride concentrations did not differ between uremic and normal apoE−/− mice (94,95). This is in contrast to our trend of decreased cholesterol and triglycerides in CKD LDLr−/− mice when compared to CTL mice regardless of diet. Despite decreased cholesterol and triglyceride levels in the VLDL and LDL fractions of CKD-HFD mice, these mice exhibited markedly accelerated atherosclerosis compared to CTL mice beginning at 12 weeks. Our CKD model is thus able to produce an increased atherosclerotic burden even in the absence of exaggerated hypercholesterolemia, hyperlipidemia, hypertension, and insulin resistance.

In our study, the aortic root and the abdominal aorta and its branches in CKD-HFD mice appear laden with a plaque that is more mature and thick, including stained sections that are more fibrotic and necrotic. Similar to our findings, Massy et al. discovered that the relative proportion of atherosclerotic lesions to lesion-free vascular tissue is increased in the aortic root of uremic apoE−/− mice when compared with controls (96). Our model did not show any changes in serum calcium and phosphate levels but did indicate increased iPTH. Plaque composition in uremic apoE−/− mice demonstrates macrophage infiltration, increased cholesterol, collagen, and calcium content than classical atherosclerosis (96). Although we did not observe more than minimal changes in serum calcium and phosphate, this model and the apoE−/− model nonetheless exhibit significantly increased vascular calcification demonstrated in prior work by other groups (59,60,96-99).

In conclusion, our study demonstrates for the first time that increased MPO levels and activity co-localizes with lesional macrophages in the artery wall in a mouse model of CKD-atherosclerosis. In addition to decreased cholinergic response in the vessel, our work suggests that MPO expression and activity may play an important role in the propagation of atherosclerotic lesions in CKD mice. However, the key limitation of this work is that the evidence is associative and does not unequivocally demonstrate the causal role of macrophage derived MPO in CKD accelerated atherosclerosis. Studies examining the effects of MPO knock-down and overexpression in macrophages in chimeric CKD mice (using MPO knock out and transgenic animals) are warranted to provide more definitive evidence on the role of macrophage derived MPO in CKD accelerated atherosclerosis.

**Experimental procedures**

**Mouse model of CKD accelerated atherosclerosis:** All animal procedures were approved by the University of Michigan Committee on Use and Care of Animals. Six-week-old male C57BL/6 LDLr−/− mice (Jackson Lab, Bar Harbor, ME) were maintained with water ad libitum and on a standard rodent diet (Lab Diet® Hudson, NH) containing 28.5% protein, 13.5% fat, 58.0% carbohydrates by calories, and 200 ppm cholesterol. These mice were housed in a climate-controlled, light-regulated facility with a 12:12 hour light-dark cycle. At age 7 weeks, the mice were subjected to either sham operation (CTL, n=20) or to 5/6 nephrectomy by removing the whole right kidney in a first procedure followed by 2/3 left kidney by renal artery ligation (CKD, n=21) after a week. At 9 weeks of age, the mice in each group were further randomly divided into two subgroups and fed on LFD containing 19.6% protein, 10.7% fat and 69.7% carbohydrates by calories or HFD containing 19.5% protein, 40.5% fat and 40.0% carbohydrates (Harlan Teklad Laboratory, Winfield, Iowa). The LFD contained 0% cholesterol, and the HFD contained 0.5% cholesterol by weight. The mice in each group, CTL-LFD, CTL-HFD, CKD-LFD, or CKD-HFD, were maintained on these diets for either 12 or 24 weeks. Evaluation of these mice was done in a blinded manner.

Murine systolic blood pressure was measured by the IITC Life Science blood pressure (tail cuff) system (Woodland Hills, CA) as described previously (100). Hematocrit was measured by CritSpin® Micro-Hematocrit centrifuge with Digital Hematocrit Reader (StatSpin® Company). HgBA1c was measured by the Helena GLYCO-Tek Affinity column method (Beaumont, Texas). Plasma iPTH was measured...
by Enzyme linked immunosorbent assay kit bought from ALPICO Diagnostics (Salem, NH).

**Lipoprotein analysis:** Plasma lipoprotein profiles were generated by fast-protein liquid chromatography (FPLC; Bio-Rad, Hercules, CA) using two Superose 6 PC 3.2/30 columns in series (GE Healthcare Life Sciences) as discussed previously (101).

**Determination of kidney function:** Plasma creatinine levels were measured by highly specific liquid chromatography electron spray ionization and tandem mass spectrometry (LC-ESI-MS/MS) as described previously (58). BUN was measured directly on IDExx Vettest 8008 Chemistry Analyzer (Westbrook, Maine) using dry slide technology.

**Atherosclerosis assessment:** Each mouse was anesthetized and perfused with phosphate buffered saline through the left ventricle followed by 3 mL of 10% buffered formalin for fixing the vascular tree. The aortic tree was removed, microdissected to remove adventitial fat, cut longitudinally, stained with Oil Red O (Sigma, St. Louis, MO) to visualize neutral lipids, and then pinned on wax plates. The images of the about 10 mm ascending and abdominal aorta were captured on a digital camera, and en face plaque quantification of total and lesional surface area was performed with computerized image analysis program (Image Pro software; Media Cybernetics, Bethesda, MD)(56). The aortic lesion ratio is derived from the ratio of Oil Red O stained area to the total surface area of en face section of the aortic tree expressed as a percentage. Aortic root cross-sections were cut from paraffin blocks of the aortic root with Leica RM 2155 Microtome and collected on glass slides. Sections were stained with H&E and Masson Trichrome for general tissue morphology and photographed with Olympus BX-51 microscope and DP-70 high resolution digital camera.

**Immunohistochemistry:** Immunohistochemistry was performed on paraffin sections with anti-mouse MPO antibody (1:500; Abcam, UK) and negative controls.

**Immunofluorescence and confocal imaging:** Snap frozen aortic sections were incubated with rabbit antibodies for anti-mouse Mac-2 (macrophage marker; 1:500; Cedarlane, NC), 3-chlorotyrosine (1:1000; Cell Sciences, MA), 3-nitrotyrosine (1:50; Abcam, UK), and o, o′-dityrosine (1:200; Cosmo Bio, CA) at 4°C overnight for immunofluorescence experiments. Appropriate negative controls were simultaneously processed to examine for background auto fluorescence. Dual immunofluorescence labeling was simultaneously scanned by an Olympus FV500 confocal laser scanning microscope, equipped with complete integrated image analysis software system (Olympus America Inc., Melville, NY).

**Oxidized amino acid quantification by mass spectrometry:** Aortic samples were analyzed as previously outlined (27) using known concentrations of isotopically labeled internal standards $^{13}$C$_6$ tyrosine, $^{13}$C$_6$ 3-nitrotyrosine, $^{13}$C$_{12}$ o,o′-dityrosine, or $^{13}$C$_8$ 3-chlorotyrosine. Oxidized amino acids were quantified by LC-ESI-MS/MS with multiple reaction monitoring MS/MS positive ion acquisition mode utilizing an Agilent 6410 triple quadrupole MS system equipped with an Agilent 1200 LC system. Labeled precursor amino acid, $^{13}$C$_9$^{15}$N$tyrosine, was added to monitor potential internal artifact formation of 3-chlorotyrosine, 3-nitrotyrosine, and o, o′-dityrosine and was noted to be negligible.

**Vascular reactivity experiments:** Mouse aortic rings were mounted in a myograph system (Danish Myo Technology A/S, Aarhus, Denmark) and reactivity was measured as previously reported (100). Force was expressed as a percent of that achieved with 80% of maximal force with phenylephrine.

**Statistical analysis:** Results are presented as the mean ± standard deviation. Differences between the groups at different time periods were considered significant at $P$ value < 0.05 using the ANOVA and Tukey–Kramer tests. For vascular reactivity studies, data were plotted using sigmoidal interpolation. Maximal steady state vasodilation values were obtained using non-linear regression and comparison between groups achieved by one-way ANOVA with Bonferroni post-hoc analysis. All analyses were made using Graph Pad Prism 7.0 (La Jolla, CA).
Myeloperoxidase oxidizes artery proteins in kidney disease

Acknowledgements: None.

Conflict of interest: The authors declare that they have no conflicts of interest with the contents of this article.

Author contributions: LZ planned, executed and conducted the experiments. AVM analyzed the corresponding results, created the figures and wrote the manuscript. JB conducted the mass spectrometry experiments. KA and FCB conducted the vascular experiments. SP planned and supervised the experiments. All authors reviewed the results, reviewed the manuscript and approved the final version of the manuscript.
Myeloperoxidase oxidizes artery proteins in kidney disease

References

1. Go, A. S., Chertow, G. M., Fan, D., McCulloch, C. E., and Hsu, C.-y. (2004) Chronic Kidney Disease and the Risks of Death, Cardiovascular Events, and Hospitalization. New England Journal of Medicine 351, 1296-1305
2. Foley, R. N., Parfrey, P. S., and Sarnak, M. J. (1998) Clinical epidemiology of cardiovascular disease in chronic renal disease. American journal of kidney diseases : the official journal of the National Kidney Foundation 32, S112-119
3. Cheung, A. K., Sarnak, M. J., Yan, G., Dwyer, J. T., Heyka, R. J., Rocco, M. V., Teehan, B. P., and Levey, A. S. (2000) Atherosclerotic cardiovascular disease risks in chronic hemodialysis patients. Kidney international 58, 353-362
4. Sarnak, M. J., Coronado, B. E., Greene, T., Wang, S. R., Kusek, J. W., Beck, G. J., and Levey, A. S. (2002) Cardiovascular disease risk factors in chronic renal insufficiency. Clinical nephrology 57, 327-335
5. Sarnak, M. J., Levey, A. S., Schoolwerth, A. C., Coresh, J., Culleton, B., Hamm, L. L., McCullough, P. A., Kasiske, B. L., Kelepouris, E., Klag, M. J., Parfrey, P., Pfeffer, M., Raij, L., Spinosa, D. J., Wilson, P. W., American Heart Association Councils on Kidney in Cardiovascular Disease, H. P. R. C. C., Epidemiology, and Prevention. (2003) Kidney disease as a risk factor for development of cardiovascular disease: a statement from the American Heart Association Councils on Kidney in Cardiovascular Disease, High Blood Pressure Research, Clinical Cardiology, and Epidemiology and Prevention. Hypertension 42, 1050-1065
6. U.S. Renal Data System USRDS 2017 Annual Data Report: Atlas of Chronic Kidney Disease and End-Stage Renal Disease in the United States. National Institutes of Health, National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD
7. Hostetter, T. H. (2004) Chronic Kidney Disease Predicts Cardiovascular Disease. N Engl J Med 351, 1344-a-1346
8. Wattanakit, K., Folsom, A. R., Selvin, E., Coresh, J., Hirsch, A. T., and Weatherley, B. D. (2007) Kidney Function and Risk of Peripheral Arterial Disease: Results from the Atherosclerosis Risk in Communities (ARIC) Study. J Am Soc Nephrol, ASN.2005111204
9. Driuêke, T. B., and Massy, Z. A. (2010) Atherosclerosis in CKD: differences from the general population. Nat Rev Nephrol 6, 723-735
10. Penno, G., Solini, A., Bonora, E., Fondelli, C., Orsi, E., Zerbini, G., Trevisan, R., Vedovato, M., Gruden, G., Cavalot, F., Cignarelli, M., Laviola, L., Morano, S., Nicolucci, A., Pugliese, G., and Group, R. I. A. C. E. R. S. (2011) Clinical significance of nonalbuminuric renal impairment in type 2 diabetes. J Hypertens 29, 1802-1809
11. Kon, V., Yang, H., and Fazio, S. (2015) Residual Cardiovascular Risk in Chronic Kidney Disease: Role of High-density Lipoprotein. Arch Med Res 46, 379-391
12. Locatelli, F., Canaud, B., Eckardt, K. U., Stenvinkel, P., Wanner, C., and Zoccali, C. (2003) Oxidative stress in end-stage renal disease: an emerging threat to patient outcome. Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association 18, 1272-1280
13. Guilgen, G., Werneck, M. L., de Noronha, L., Martins, A. P., Varela, A. M., Nakao, L. S., and Pecoits-Filho, R. (2011) Increased calcification and protein nitr ation in arteries of chronic kidney disease patients. Blood Purf 32, 296-302
14. Vaziri, N. D. (2004) Oxidative stress in uremia: Nature, mechanisms, and potential consequences. Seminars in Nephrology 24, 469-473
15. Vaziri, N. D. (2004) Roles of oxidative stress and antioxidant therapy in chronic kidney disease and hypertension. Curr Opin Nephrol Hypertens 13, 93-99
16. Landmesser, U., and Drexler, H. (2003) Oxidative stress, the renin-angiotensin system, and atherosclerosis. European Heart Journal Supplements 5, A3-A7
Myeloperoxidase oxidizes artery proteins in kidney disease

17. Klebanoff, S. J. (1980) Oxygen metabolism and the toxic properties of phagocytes. *Ann. Intern. Med.* **93**, 480-489

18. Ross, R. (1999) Atherosclerosis—an inflammatory disease. *N Engl J Med* **340**, 115-126.

19. Brown, M. S., and Goldstein, J. L. (1992) Koch's postulates for cholesterol. *Cell* **71**, 187-188

20. Goldstein, J. L., Ho, Y. K., Basu, S. K., and Brown, M. S. (1979) Binding site on macrophages that mediates uptake and degradation of acetylated low density lipoprotein, producing massive cholesterol deposition. *Proc Natl Acad Sci U S A* **76**, 333-337

21. Witztum, J. L., and Steinberg, D. (1991) Role of oxidized low density lipoprotein in atherogenesis. *J Clin Invest.* **88**, 1785-1792

22. Steinberg, D. (2002) Atherogenesis in perspective: hypercholesterolemia and inflammation as partners in crime. *Nat Med* **8**, 1211-1217

23. Haberland, M. E., Fong, D., and Cheng, L. (1988) Malondialdehyde-altered protein occurs in atheroma of Watanabe heritable hyperlipidemic rabbits. *Science* **241**, 215-218

24. Yla-Herttuala, S., Palinski, W., Butler, S. W., Picard, S., Steinberg, D., and Witztum, J. L. (1994) Rabbit and human atherosclerotic lesions contain IgG that recognizes epitopes of oxidized LDL. *Arterioscler Thromb* **14**, 32-40

25. Yla-Herttuala, S., Palinski, W., Rosenfeld, M. E., Parthasarathy, S., Carew, T. E., Butler, S., Witztum, J. L., and Steinberg, D. (1989) Evidence for the presence of oxidatively modified low density lipoprotein in atherosclerotic lesions of rabbit and man. *J Clin Invest* **84**, 1086-1095

26. Klebanoff, S. J., and Rosen, H. (1978) The role of myeloperoxidase in the microbicidal activity of polymorphonuclear leukocytes. *Ciba Found Symp*, 263-284

27. Vivekanandan-Giri, A., Byun, J., and Pennathur, S. (2011) Quantitative analysis of amino acid oxidation markers by tandem mass spectrometry. *Methods in enzymology* **491**, 73-89

28. Hazen, S. L., and Heinecke, J. W. (1997) 3-Chlorotyrosine, a specific marker of myeloperoxidase-catalyzed oxidation, is markedly elevated in low density lipoprotein isolated from human atherosclerotic intima. *J Clin Invest* **99**, 2075-2081

29. Eiserich, J. P., Hristova, M., Cross, C. E., Jones, A. D., Freeman, B. A., Halliwell, B., and van der Vliet, A. (1998) Formation of nitric oxide-derived inflammatory oxidants by myeloperoxidase in neutrophils. *Nature* **391**, 393-397

30. McCormick, M. L., Gaut, J. P., Lin, T. S., Britigan, B. E., Buettner, G. R., and Heinecke, J. W. (1998) Electron paramagnetic resonance detection of free tyrosyl radical generated by myeloperoxidase, lactoperoxidase, and horseradish peroxidase. *J Biol Chem* **273**, 32030-32037

31. Brennan, M. L., Wu, W., Fu, X., Shen, Z., Song, W., Frost, H., Vadseth, C., Marine, L., Lenkiewicz, E., Borchers, M. T., Lusis, A. J., Lee, J. J., Lee, N. A., Abu-Soud, H. M., Ischiropoulos, H., and Hazen, S. L. (2002) A tale of two controversies: defining both the role of peroxides in nitrotyrosine formation in vivo using eosinophil peroxidase and myeloperoxidase-deficient mice, and the nature of peroxidase-generated reactive nitrogen species. *J Biol Chem* **277**, 17415-17427

32. Abu-Soud, H. M., and Hazen, S. L. (2000) Nitric oxide is a physiological substrate for mammalian peroxidases. *J Biol Chem* **275**, 37524-37532

33. Daugherty, A., Dunn, J. L., Rateri, D. L., and Heinecke, J. W. (1994) Myeloperoxidase, a catalyst for lipoprotein oxidation, is expressed in human atherosclerotic lesions. *The Journal of Clinical Investigation* **94**, 437-444

34. Hazell, L. J., Arnold, L., Flowers, D., Waeg, G., Malle, E., and Stocker, R. (1996) Presence of hypochlorite-modified proteins in human atherosclerotic lesions. *The Journal of Clinical Investigation* **97**, 1535-1544

35. Leeuwenburgh, C., Hardy, M. M., Hazen, S. L., Wagner, P., Oh-ishi, S., Steinbrecher, U. P., and Heinecke, J. W. (1997) Reactive nitrogen intermediates promote low density lipoprotein oxidation in human atherosclerotic intima. *J Biol Chem* **272**, 1433-1436

36. Heller, J. L., Crowley, J. R., Hazen, S. L., Salvay, D. M., Wagner, P., Pennathur, S., and Heinecke, J. W. (2000) p-Hydroxyphenylacetaldehyde, an Aldehyde Generated by
Myeloperoxidase, Modifies Phospholipid Amino Groups of Low Density Lipoprotein in Human Atherosclerotic Intima. *Journal of Biological Chemistry* **275**, 9957-9962

37. Brennan, M. L., Penn, M. S., Van Lente, F., Nambi, V., Shishehbor, M. H., Aviles, R. J., Goormastic, M., Pepoy, M. L., McErlean, E. S., Topol, E. J., Nissen, S. E., and Hazen, S. L. (2003) Prognostic value of myeloperoxidase in patients with chest pain. *N Engl J Med* **349**, 1595-1604

38. Pennathur, S., Wagner, J. D., Leeuwenburgh, C., Litwak, K. N., and Heinkecke, J. W. (2001) A hydroxyl radical-like species oxidizes cynomolgus monkey artery wall proteins in early diabetic vascular disease. *The Journal of Clinical Investigation* **107**, 853-860

39. Baldus, S., Eisnerich, J. P., Brennan, M. L., Jackson, R. M., Alexander, C. B., and Freeman, B. A. (2002) Spatial mapping of pulmonary and vascular nitrotyrosine reveals the pivotal role of myeloperoxidase as a catalyst for tyrosine nitration in inflammatory diseases. *Free Radic Biol Med* **33**, 1010

40. Duzguncinar, O., Yazuz, B., Hazirolan, T., Deniz, A., Tokgozoglu, S. L., Akata, D., and Demirpence, E. (2008) Plasma myeloperoxidase is related to the severity of coronary artery disease. *Acta Cardiol* **63**, 147-152

41. Shishehbor, M. H., Aviles, R. J., Brennan, M. L., Fu, X., Goormastic, M., Pearce, G. L., Gokce, N., Keaney, J. F., Penn, M. S., Sprecher, D. L., Vita, J. A., and Hazen, S. L. (2003) Association of nitrotyrosine levels with cardiovascular disease and modulation by statin therapy. *JAMA* **289**, 1675-1680

42. Shishehbor, M. H., Brennan, M. L., Aviles, R. J., Fu, X., Penn, M. S., Sprecher, D. L., and Hazen, S. L. (2003) Statins promote potent systemic antioxidant effects through specific inflammatory pathways. *Circulation* **108**, 426-431

43. Shishehbor, M. H., and Hazen, S. L. (2004) Inflammatory and oxidative markers in atherosclerosis: relationship to outcome. *Curr Atheroscler Rep* **6**, 243-250

44. Vivekanandan-Giri, A., Slocum, J. L., Byun, J., Tang, C., Sands, R. L., Gillespie, B. W., Heinecke, J. W., Saran, R., Kaplan, M. J., and Pennathur, S. (2013) High density lipoprotein is targeted for oxidation by myeloperoxidase in rheumatoid arthritis. *Annals of the rheumatic diseases* **72**, 1725-1731

45. Smith, C. K., Vivekanandan-Giri, A., Tang, C., Knight, J. S., Mathew, A., Padilla, R. L., Gillespie, B. W., Carmona-Rivera, C., Liu, X., Subramanian, V., Hasni, S., Thompson, P. R., Heinecke, J. W., Saran, R., Pennathur, S., and Kaplan, M. J. (2014) Neutrophil extracellular trap-derived enzymes oxidize high-density lipoprotein: an additional proatherogenic mechanism in systemic lupus erythematosus. *Arthritis Rheumatol* **66**, 2523-2544

46. Wang, Z., Nicholls, S. J., Rodriguez, E. R., Kummu, O., Horkko, S., Barnard, J., Reynolds, W. E., Topol, E. J., DiDonato, J. A., and Hazen, S. L. (2007) Protein carbamylation links inflammation, smoking, uremia and atherogenesis. *Nat Med* **13**, 1176-1184

47. Jaisson, S., Pietremont, C., and Gillery, P. (2011) Carbamylation-derived products: bioactive compounds and potential biomarkers in chronic renal failure and atherosclerosis. *Clin Chem* **57**, 1499-1505

48. Tang, W. H., Shrestha, K., Wang, Z., Borowski, A. G., Troughton, R. W., Klein, A. L., and Hazen, S. L. (2013) Protein carbamylation in chronic systolic heart failure: relationship with renal impairment and adverse long-term outcomes. *Journal of cardiac failure* **19**, 219-224

49. Koeth, R. A., Kalantar-Zadeh, K., Wang, Z., Fu, X., Tang, W. H., and Hazen, S. L. (2013) Protein carbamylation predicts mortality in ESRD. *J Am Soc Nephrol* **24**, 853-861

50. Wang, A. Y., Lam, C. W., Chan, I. H., Wang, M., Lui, S. F., and Sanderson, J. E. (2010) Prognostic value of plasma myeloperoxidase in ESRD patients. *American journal of kidney diseases : the official journal of the National Kidney Foundation* **56**, 937-946

51. Kisic, B., Miric, D., Dragojevic, I., Rasic, J., and Popovic, L. (2016) Role of Myeloperoxidase in Patients with Chronic Kidney Disease. *Oxid Med Cell Longev* **2016**, 1069743
Myeloperoxidase oxidizes artery proteins in kidney disease

52. Bansal, V., Cunanan, J., Hoppensteadt, D., Jeske, W., and Fareed, J. (2007) Hemodialysis Mediated Upregulation of Myeloperoxidase in End Stage Renal Disease: Pathophysiologic Implications. *The FASEB Journal* 21, A438-A439

53. Madhusudhana Rao, A., Anand, U., and Anand, C. V. (2011) Myeloperoxidase in Chronic Kidney Disease. *Indian Journal of Clinical Biochemistry* 26, 28-31

54. Afshinnia, F., Zeng, L., Byun, J., Gadgebeku, C. A., Magnone, M. C., Whatling, C., Valastro, B., Kretzler, M., Pennathur, S., and Michigan Kidney Translational Core, C. I. G. (2017) Myeloperoxidase Levels and Its Product 3-Chlorotyrosine Predict Chronic Kidney Disease Severity and Associated Coronary Artery Disease. *Am J Nephrol* 46, 73-81

55. Pennathur, S., Jackson-Lewis, V., Przedborski, S., and Heinecke, J. W. (1999) Mass spectrometric quantification of 3-nitrotyrosine, ortho-tyrosine, and o,o′-dityrosine in brain tissue of 1-methyl-4-phenyl-1,2,3, 6-tetrahydropyridine-treated mice, a model of oxidative stress in Parkinson's disease. *J Biol Chem* 274, 34621-34628

56. Brennan, M.-L., Anderson, M. M., Shih, D. M., Qu, X.-D., Wang, X., Mehta, A. C., Lim, L. L., Shi, W., Hazen, S. L., Jacob, J. S., Crowley, J. R., Heinecke, J. W., and Lusis, A. J. (2001) Increased atherosclerosis in myeloperoxidase-deficient mice. *The Journal of Clinical Investigation* 107, 419-430

57. McMillen, T. S., Heinecke, J. W., and LeBoeuf, R. C. (2005) Expression of human myeloperoxidase by macrophages promotes atherosclerosis in mice. *Circulation* 111, 2798-2804

58. Mathew, A. V., Zeng, L., Byun, J., and Pennathur, S. (2015) Metabolomic Profiling of Arginine Metabolome Links Altered Methylation to Chronic Kidney Disease Accelerated Atherosclerosis. *J Proteomics Bioinform Suppl 14*

59. Davies, M. R., and Hruska, K. A. (2001) Pathophysiological mechanisms of vascular calcification in end-stage renal disease. *Kidney international* 60, 472-479

60. Davies, M. R., Lund, R. J., and Hruska, K. A. (2003) BMP-7 is an efficacious treatment of vascular calcification in a murine model of atherosclerosis and chronic renal failure. *J Am Soc Nephrol* 14, 1559-1567

61. Bisgaard, L. S., Bosteen, M. H., Fink, L. N., Sorensen, C. M., Rosendahl, A., Mogensen, C. K., Rasmussen, S. E., Rolin, B., Nielsen, L. B., and Pedersen, T. X. (2016) Liraglutide Reduces Both Atherosclerosis and Kidney Inflammation in Moderately Uremic LDLr−/− Mice. *PLoS One* 11, e0168396

62. Pennathur, S., Vivekanandan-Giri, A., Locy, M. L., Kulkarni, T., Zhi, D., Zeng, L., Byun, J., de Andrade, J. A., and Thannickal, V. J. (2016) Oxidative Modifications of Protein Tyrosyl Residues Are Increased in Plasma of Human Subjects with Interstitial Lung Disease. *American Journal of Respiratory and Critical Care Medicine* 193, 861-868

63. Leeuwenburgh, C., Rasmussen, J. E., Hsu, F. F., Mueller, D. M., Pennathur, S., and Heinecke, J. W. (1997) Mass spectrometric quantification of markers for protein oxidation by tyrosyl radical, copper, and hydroxyl radical in low density lipoprotein isolated from human atherosclerotic plaques. *The Journal of biological chemistry* 272, 3520-3526

64. Zheng, L., Nukuna, B., Brennan, M. L., Sun, M., Goormastic, M., Settle, M., Schmitt, D., Fu, X., Thomson, L., Fox, P. L., Ischiropoulos, H., Smith, J. D., Kinter, M., and Hazen, S. L. (2004) Apolipoprotein A-I is a selective target for myeloperoxidase-catalyzed oxidation and functional impairment in subjects with cardiovascular disease. *J Clin Invest* 114, 529-541

65. Zalba, G., Fortuno, A., and Diez, J. (2006) Oxidative stress and atherosclerosis in early chronic kidney disease. *Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association* 21, 2686-2690

66. Gluba-Brzozka, A., Michalska-Kasiczak, M., Franczyk, B., Nocun, M., Toth, P., Banach, M., and Rysz, J. (2016) Markers of increased atherosclerotic risk in patients with chronic kidney disease: a preliminary study. *Lipids Health Dis* 15, 22

67. Kon, V., Linton, M. F., and Fazio, S. (2011) Atherosclerosis in chronic kidney disease: the role of macrophages. *Nat Rev Nephrol* 7, 45-54
Chumakov, A. M., Chumakova, E. A., Chih, D., and Koeffler, H. P. (2000) Molecular analysis of the human myeloperoxidase promoter region. *International journal of oncology* **16**, 401-411

Asselbergs, F. W., Reynolds, W. F., Cohen-Tervaert, J. W., Jessurun, G. A., and Tio, R. A. (2004) Myeloperoxidase polymorphism related to cardiovascular events in coronary artery disease. *Am J Med* **116**, 429-430

Nikpoor, B., Turecki, G., Fournier, C., Theroux, P., and Rouleau, G. A. (2001) A functional myeloperoxidase polymorphic variant is associated with coronary artery disease in French-Canadians. *Am Heart J* **142**, 336-339

Grahl, D. A., Axelsson, J., Nordfors, L., Heimburger, O., Barany, P., Gao, Y. Z., Qureshi, A. R., Kato, S., Watanabe, M., Suliman, M., Riella, M. C., Lindholm, B., Stenvinkel, P., and Pecoits-Filho, R. (2007) Associations between the CYBA 242C/T and the MPO -463G/A polymorphisms, oxidative stress and cardiovascular disease in chronic kidney disease patients. *Blood Purif* **25**, 210-218

Pecoits-Filho, R., Stenvinkel, P., Marchlewska, A., Heimburger, O., Barany, P., Hoff, C. M., Holmes, C. J., Suliman, M., Lindholm, B., Schalling, M., and Nordfors, L. (2003) A functional variant of the myeloperoxidase gene is associated with cardiovascular disease in end-stage renal disease patients. *Kidney Int Suppl* **S172-S176

Piedrafita, F. J., Molander, R. B., Vansant, G., Orlova, E. A., Pfahl, M., and Reynolds, W. F. (1996) An Alu element in the myeloperoxidase promoter contains a composite SP1-thyroid hormone-retinoic acid response element. *J Biol Chem* **271**, 14412-14420

Kumar, A. P., Piedrafita, F. J., and Reynolds, W. F. (2004) Peroxisome proliferator-activated receptor gamma ligands regulate myeloperoxidase expression in macrophages by an estrogen-dependent mechanism involving the -463GA promoter polymorphism. *J Biol Chem* **279**, 8300-8315

Kumar, A. P., and Reynolds, W. F. (2005) Statins downregulate myeloperoxidase gene expression in macrophages. *Biochem Biophys Res Commun* **331**, 442-451

Reynolds, W. F., Kumar, A. P., and Piedrafita, F. J. (2006) The human myeloperoxidase gene is regulated by LXR and PPARalpha ligands. *Biochem Biophys Res Commun* **349**, 846-854

Castellani, L. W., Chang, J. J., Wang, X., Lusis, A. J., and Reynolds, W. F. (2006) Transgenic mice express human MPO -463G/A alleles at atherosclerotic lesions, developing hyperlipidemia and obesity in -463G males. *J Lipid Res.* **47**, 1366-1377

Cayley, W. E., Jr. (2004) Prognostic value of myeloperoxidase in patients with chest pain. *N Engl J Med* **350**, 516-518; author reply 516-518

Doi, K., Noiri, E., Maeda, R., Nakao, A., Kobayashi, S., Tokunaga, K., and Fujita, T. (2007) Functional polymorphism of the myeloperoxidase gene in hypertensive nephrosclerosis dialysis patients. *Hypertens Res* **30**, 1193-1198

Bouali, H., Nietert, P., Nowling, T. M., Pandey, J., Dooley, M. A., Cooper, G., Harley, J., Kamen, D. L., Oates, J., and Gilkeson, G. (2007) Association of the G-463A myeloperoxidase gene polymorphism with renal disease in African Americans with systemic lupus erythematosus. *J Rheumatol* **34**, 2028-2034

Bro, S., Bentzon, J. F., Falk, E., Andersen, C. B., Olgaard, K., and Nielsen, L. B. (2003) Chronic renal failure accelerates atherogenesis in apolipoprotein E-deficient mice. *J Am Soc Nephrol* **14**, 2466-2474

Malyszko, J. (2010) Mechanism of endothelial dysfunction in chronic kidney disease. *Clinica chimica acta; international journal of clinical chemistry* **411**, 1412-1420

Yamamoto, Y., Yamashita, T., Kitagawa, F., Sakamoto, K., Giddings, J. C., and Yamamoto, J. (2010) The effect of the long term aspirin administration on the progress of atherosclerosis in apoE/-/- LDLR/-/- double knockout mouse. *Thrombosis research* **125**, 246-252

Csányi, G., Gajda, M., Franczyk-Zarow, M., Kostogrys, R., Gwoźdz, P., Mateuszuk, L., Sternak, M., Wojcik, L., Zalewska, T., Walski, M., and Chlopicki, S. (2012) Functional alterations in
Myeloperoxidase oxidizes artery proteins in kidney disease

85. Wolfle, S. E., and de Wit, C. (2005) Intact endothelium-dependent dilation and conducted responses in resistance vessels of hypercholesterolemic mice in vivo. *Journal of vascular research* **42**, 475-482

86. Ketonen, J., and Mervaala, E. (2008) Effects of dietary sodium on reactive oxygen species formation and endothelial dysfunction in low-density lipoprotein receptor-deficient mice on high-fat diet. *Heart and vessels* **23**, 420-429

87. Meyrelles, S. S., Peotta, V. A., Pereira, T. M., and Vasquez, E. C. (2011) Endothelial dysfunction in the apolipoprotein E-deficient mouse: insights into the influence of diet, gender and aging. *Lipids Health Dis* **10**, 211

88. Wu-Wong, J. R., Li, X., and Chen, Y. W. (2015) Different vitamin D receptor agonists exhibit differential effects on endothelial function and aortic gene expression in 5/6 nephrectomized rats. *The Journal of steroid biochemistry and molecular biology* **148**, 202-209

89. Eraranta, A., Tormanen, S., Koob, P., Vehmas, T. I., Lakkisto, P., Tikkanen, I., Moilanen, E., Niemela, O., Mustonen, J., and Porsti, I. (2014) Phosphate binding reduces aortic angiotensin-converting enzyme and enhances nitric oxide bioactivity in experimental renal insufficiency. *Am J Nephrol* **39**, 400-408

90. Bro, S., Borup, R., Andersen, C. B., Moeller, F., Olgaard, K., and Nielsen, L. B. (2006) Uremia-specific effects in the arterial media during development of uremic atherosclerosis in apolipoprotein E-deficient mice. *Arterioscler Thromb Vasc Biol* **26**, 570-575

91. Bro, S., Binder, C. J., Witzum, J. L., Olgaard, K., and Nielsen, L. B. (2007) Inhibition of the renin-angiotensin system abolishes the proatherogenic effect of uremia in apolipoprotein E-deficient mice. *Arterioscler Thromb Vasc Biol* **27**, 1080-1086

92. Bro, S., Flyvbjerg, A., Binder, C. J., Bang, C. A., Denner, L., Olgaard, K., and Nielsen, L. B. (2008) A neutralizing antibody against receptor for advanced glycation end products (RAGE) reduces atherosclerosis in uremic mice. *Atherosclerosis* **201**, 274-280

93. Bro, S., Bollano, E., Brüel, A., Olgaard, K., and Nielsen, L. B. (2008) Cardiac structure and function in a mouse model of uremia without hypertension. *Scand J Clin Lab Invest* **68**, 660-666

94. Bro, S. (2009) Cardiovascular effects of uremia in apolipoprotein E-deficient mice. *Dan Med Bull* **56**, 177-192

95. Bro, S., Bentzon, J. F., Falk, E., Andersen, C. B., Olgaard, K., and Nielsen, L. B. (2003) Chronic renal failure accelerates atherogenesis in apolipoprotein E-deficient mice. *J Am Soc Nephrol* **14**, 2466-2474

96. Massy, Z. A., Ivanovski, O., Nguyen-Khoa, T., Angulo, J., Szumilak, D., Mothu, N., Phan, O., Daudon, M., Lacour, B., Drüeke, T. B., and Muntzel, M. S. (2005) Uremia accelerates both atherosclerosis and arterial calcification in apolipoprotein E knockout mice. *J Am Soc Nephrol* **16**, 109-116

97. Phan, O., Ivanovski, O., Nguyen-Khoa, T., Mothu, N., Angulo, J., Westenfeld, R., Ketteler, M., Meert, N., Maizel, J., Nikolov, I. G., Vanholder, R., Lacour, B., Drueke, T. B., and Massy, Z. A. (2005) Sevelamer prevents uremia-enhanced atherosclerosis progression in apolipoprotein E-deficient mice. *Circulation* **112**, 2875-2882

98. Phan, O., Ivanovski, O., Nikolov, I. G., Joki, N., Maizel, J., Louvet, L., Chasseraud, M., Nguyen-Khoa, T., Lacour, B., Drueke, T. B., and Massy, Z. A. (2008) Effect of oral calcium carbonate on aortic calcification in apolipoprotein E-deficient (apoE-/-) mice with chronic renal failure. *Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association* **23**, 82-90

99. Shobeiri, N., Adams, M. A., and Holden, R. M. (2010) Vascular Calcification in Animal Models of CKD: A Review. *American Journal of Nephrology* **31**, 471-481
Myeloperoxidase oxidizes artery proteins in kidney disease

Atkins, K. B., Seki, Y., Saha, J., Eichinger, F., Charron, M. J., and Brosius, F. C. (2015) Maintenance of GLUT4 expression in smooth muscle prevents hypertension-induced changes in vascular reactivity. *Physiological Reports* 3

Gerdes, L. U., Gerdes, C., Klausen, I. C., and Faergeman, O. (1992) Generation of analytic plasma lipoprotein profiles using two prepacked superose 6B columns. *Clinica Chimica Acta* 205, 1-9
FOOTNOTES

Funding was provided by the National Institute of Diabetes and Digestive and Kidney Diseases (P30DK081943 and P30DK089503) to S.P, National Heart, Lung, and Blood Institute (K08HL130944) to A.V.M, as well as grants from the Renal Research Institute to K.A.

The abbreviations used are: MPO, myeloperoxidase; CKD, chronic kidney disease; CVD, cardiovascular disease; CTL, control; LFD, low fat diet; HFD, high fat-high cholesterol diet; LDL, low density lipoprotein; VLDL, very low density lipoprotein; HDL, high density lipoprotein; LDLr-/-, LDL receptor deficient mice; NO2-, nitrogen dioxide radical; apoE-/-, apolipoprotein E deficient mice; O2-, superoxide; ONOO-, peroxynitrate; ESRD, end stage renal disease; BUN, serum blood urea nitrogen; iPTH, intact parathyroid hormone; HbA1c, hemoglobin A1c; H&E, hematoxylin & eosin; MS, mass spectrometry; BSA, bovine serum albumin.
Myeloperoxidase oxidizes artery proteins in kidney disease

Tables

Table 1: Biological characteristics of the four groups of mice

Male mice (N=6-12 each group) were utilized for this study; CTL; control LDLr−/− mice; CKD; 5/6 nephrectomized LDLr−/− mice; LFD; low fat diet; HFD; high fat- high cholesterol diet; BUN; Blood urea nitrogen; iPTH; intact parathyroid hormone.

* p value <0.05 compared to mice in the same group of different ages;
# p value <0.05 CKD mice compared to controls within same diet group;
% p value <0.05 compared to mice fed low fat diet with similar renal function.

| Biological parameters(units) | Weeks | CTL-LFD | CKD-LFD | CTL-HFD | CKD-HFD |
|-----------------------------|-------|---------|---------|---------|---------|
| **Body Weight (g)**        |       |         |         |         |         |
| 0                           | 22.6±0.4 | 22.2±0.3 | 22.6±0.3 | 22.2±0.3 |
| 12                          | 32.1±0.9* | 27.7±0.4** | 34.6±0.9* | 28.7±0.5 ## |
| 24                          | 34.5±0.9* | 30.3±0.9## | 39.3±1.4* | 33.1±1.0 ##% |
| **Systolic Blood Pressure (mmHg)** |     |         |         |         |         |
| 0                           | 103.0±4.9 | 110.4±4.8 | 110.4±3.9 | 111.7±5.4 |
| 12                          | 102.7±4.1 | 99.7±2.4  | 101.8±4.6 | 104.0±6.2 |
| 24                          | 108.9±4.4 | 89.70±3.2 | 108.2±3.4 | 91.8±4.4  |
| **Glycohemoglobin (%)**    |       |         |         |         |         |
| 0                           | 7.1±0.4  | 6.7±0.2  | 6.6±0.2  | 7.0±0.5  |
| 24                          | 6.9±0.2  | 6.5±0.2  | 6.4±0.1  | 7.0±0.3  |
| **Hematocrit (%)**         |       |         |         |         |         |
| 0                           | 60.8±0.9 | 57.4±0.7 | 61.7±0.5 | 57.1±0.8 |
| 12                          | 61.7±0.4 | 54.0±0.4 ## | 59.8±0.5 | 53.5±0.7 ## |
| 24                          | 59.6±0.7 | 51.6±0.8 ## | 59.0±0.6 | 51.9±0.8 ## |
| **BUN(mg/dL)**              |       |         |         |         |         |
| 0                           | 22.6±1.5 | 43.7±1.4 # | 27.0±1.7 | 44.7±3.4 # |
| 24                          | 29.5±1.5 | 43.7±1.4 # | 27.0±1.7 | 44.7±3.4 # |
| **Creatinine (mg/dL)**      |       |         |         |         |         |
| 0                           | 0.06±0.00 | 0.21±0.01 # | 0.08±0.00 | 0.20±0.01 # |
| 12                          | 0.08±0.00 | 0.20±0.01 # | 0.08±0.03 | 0.20±0.01 # |
| 24                          | 0.12±0.01 | 0.24±0.02 # | 0.11±0.01 | 0.22±0.05 # |
| **Calcium (mg/dL)**         |       |         |         |         |         |
| 0                           | 10.4±0.4  | 9.9±1.1  | 9.8±0.6  | 10.1±0.7 |
| 24                          | 9.8±0.1  | 10.5±0.3 | 10.3±1.0 | 10.1±0.5 |
| **Phosphorus (mg/dL)**      |       |         |         |         |         |
| 0                           | 10.3±2.5  | 11.6±3.2 | 11.7±1.5 | 11.1±1.0 |
| 24                          | 9.7±1.6  | 9.6±1.1  | 9.7±1.1  | 10.1±1.0 |
| **Plasma iPTH (pg/mL)**     |       |         |         |         |         |
| 0                           | 336±65   | 341±124 | 366±98  | 368±136 |
| 24                          | 314±7    | 594±8## | 329±7   | 700±6## |
| **Plasma Cholesterol (mg/dL)** |     |         |         |         |         |
| 0                           | 249.2±8.1 | 281.4±25.5 | 278.2±11.7 | 290.1±27.2 |
| 12                          | 389.9±212.0 | 282.8±49.0## | 1712±937.4##% | 1478±599.6##% |
| 24                          | 857.7±93.4* | 471±53.1## | 2432±207.8##% | 1532±244.1##% |
| **Plasma Triglyceride (mg/dL)** |   |         |         |         |         |
| 0                           | 198.5±9.8 | 263.6±17.2 | 235.4±35.6 | 216.6±21.9 |
| 12                          | 186.4±25.7 | 412.6±239.2 | 231.2±79.0 | 368.3±135.1 |
| 24                          | 389.4±66.2* | 308±16.8## | 1094±147.5##% | 668.2±77.2##% |
Figure 1: 5/6 nephrectomized mice have lower cholesterol and triglyceride content in the VLDL and LDL fractions

Fast Protein Liquid Chromatography fractions of pooled plasma from the four groups of male LDLr−/− control (CTL) and 5/6 nephrectomized (CKD) mice on high fat-high cholesterol diet (HFD) and low fat diet (LFD) for 24 weeks reveal that CKD mice have lower VLDL and LDL cholesterol levels (A) with similar HDL cholesterol levels compared to CTL mice of the same diet. Similarly, the VLDL and LDL triglyceride levels (B) are lower in CKD mice when compared to CTL mice of the same diet. On the whole, mice on LFD had lower VLDL and LDL triglyceride and cholesterol content than mice on the HFD.
Figure 2: 5/6 nephrectomy (CKD) mice on high fat-high cholesterol diet (HFD) demonstrate increased atherosclerosis compared to control mice on HFD and CKD mice on low fat diet. Representative Oil red staining and morphometry of aorta and main branches from male LDLr⁻/⁻ mice. A, F: Control (CTL) mice on low fat diet (LFD) for 12 and 24 weeks; B, G: 5/6 nephrectomized (CKD) mice on LFD for 12 and 24 weeks. C, H: CTL mice on high fat-high cholesterol diet (HFD) for 12 and 24 weeks; D, I: CKD mice on HFD for 12 and 24 weeks; and E and J: Analysis of total atherosclerotic lesion area ratio between the four groups at 12 weeks and 24 weeks reveals increases in lesional ratio (ratio of Oil red O stained area to the total surface area of en-face section of the aortic tree expressed as a percentage) in the CKD and HFD groups. The CKD HFD group shows the highest lesional area among the 4 groups and significantly higher when compared with CTL HFD group. (*p<0.05; number of mice in parenthesis).
Figure 3: 5/6 nephrectomy mice on high fat-high cholesterol diet have advanced atherosclerotic lesions

Representative cross sections at aorta root from male LDLr⁻/⁻ mice stained with hematoxylin and eosin (H&E; A and B) and Masson Trichrome (C and D). A and C: 5/6 nephrectomized (CKD) mice on high fat-high cholesterol diet (HFD) for 24 weeks; B and D: CKD mice on HFD for 24 weeks. Magnification 400x. The atherosclerotic lesions from CKD mice on HFD had elevated aortic plaque area fraction, luminal narrowing, macrophage filtration and fibrosis.
Myeloperoxidase oxidizes artery proteins in kidney disease

Figure 4: 5/6 nephrectomy mice on high fat-high cholesterol diet demonstrate decreased response to acetylcholine in aortic rings
Response to acetylcholine in pre-contracted (phenylephrine) aortic rings from male LDLr<sup>−/−</sup> control (CTL) and 5/6 nephrectomized (CKD) on 24 weeks of low fat diet (LFD) and high fat-high cholesterol diet (HFD): CTL-LFD (Emax: 5.804±3.296%), CKD-LFD (Emax: 13.39±2.941%), and CTL-HFD (Emax: 13.01±2.295%) mice were similar. In contrast, cholinergic responsiveness was significantly decreased in aortic rings from CKD-HFD (32.48±3.126%) mice (Emax: *p<0.01 vs. CKD-LFD/CTL-HFD; *p<0.0001 vs. CTL-LFD; n=5/group).
Myeloperoxidase oxidizes artery proteins in kidney disease

Figure 5: Increased myeloperoxidase and its products present in atherosclerotic lesions of 5/6 nephrectomy mice on high fat-high cholesterol diet.

Representative immunohistologic staining for myeloperoxidase (MPO) in the aortic cross sections from male LDLr<sup>−/−</sup> mice after 24 weeks of high fat-high cholesterol diet (HFD). Magnification 1000x. Intense staining for MPO was observed in the intimal lesions of 5/6 nephrectomized (CKD-HFD) mice (B) atherosclerotic lesions when compared to control (CTL-HFD) mice (A). Mass spectrometric quantification of oxidized amino acids in aortic proteins in male LDLr<sup>−/−</sup> mice on 24 weeks of HFD or low fat diet (LFD): C; 3-chlorotyrosine, D; 3-nitrotyrosine, and E; o,o′-dityrosine expressed as ratios to precursor amino acid tyrosine in µmol/mol (n=4-7 each); Panels F to H show the correlation using Pearson’s correlation between the three oxidation products (*p<0.05).
Figure 6: Macrophages, myeloperoxidase and myeloperoxidase products co-localize in the arterial wall of 5/6 nephrectomized mice on high fat-high cholesterol diet
Representative immunofluorescence and double labeling in the aortic cross sections from male LDLr⁻/⁻ 5/6 nephrectomized mice on 24 weeks of high fat-high cholesterol diet for Mac-2 (macrophage marker), myeloperoxidase, 3-chlorotyrosine, 3-nitrotyrosine and o,o'-dityrosine. Section view of the aortic wall double labeled for Mac-2 (green; E to H) and MPO (red, A), 3-chlorotyrosine (red, B), 3-nitrotyrosine (red, C) and o,o'-dityrosine (red, D). In all sections, the signals of MPO, o, o'-dityrosine, 3-nitrotyrosine, and 3-chlorotyrosine co-localized with Mac-2 (yellow, I to L).
Myeloperoxidase derived oxidants damage artery wall proteins in an animal model of chronic kidney disease accelerated atherosclerosis

Lixia Zeng, Anna V. Mathew, Jaeman Byun, Kevin B Atkins, Frank C Brosius III and Subramaniam Pennathur

*J. Biol. Chem.* published online March 26, 2018

Access the most updated version of this article at doi: 10.1074/jbc.RA117.000559

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts