Mitochondrial DNA Damage can Promote Atherosclerosis Independently of Reactive Oxygen Species through Effects on Smooth Muscle Cells and Monocytes, and Correlates with Higher Risk Plaques in Humans

Running title: Yu et al.; Mitochondrial DNA damage in atherosclerosis

Emma Yu, MD1; Patrick A. Calvert, MD, PhD1,2; John R. Mercer, PhD1; James Harrison1; Lauren Baker1; Nichola L. Figg1; Sheetal Kumar1; Julie J. Wang, PhD1; Liam A. Hurst, MSc1; Daniel R. Obaid, MD1,2; Angela Logan, PhD3; Nick E. J. West, MD2; Murray C. H. Clarke, PhD1; Antonio Vidal-Puig, PhD1; Michael P. Murphy, PhD3; Martin R. Bennett, MD, PhD1,2

1Division of Cardiovascular Medicine, University of Cambridge; 2Dept of Cardiology, Papworth Hospital; 3MRC Mitochondrial Biology Unit; 4Institute of Metabolic Sciences, University of Cambridge, Cambridge, United Kingdom

Address for Correspondence:
Martin R. Bennett, MD, PhD
Division of Cardiovascular Medicine, University of Cambridge
Addenbrooke’s Centre for Clinical Investigation, PO Box 110
Addenbrooke’s Hospital
Cambridge, CB2 2QQ, UK
Tel: 44-1223-331504
Fax: 44-1223-331505
Email: mrb@mole.bio.cam.ac.uk

Abstract:

Background—Mitochondrial DNA (mtDNA) damage occurs in both circulating cells and the vessel wall in human atherosclerosis. However it is unclear whether mtDNA damage directly promotes atherogenesis or is a consequence of tissue damage, which cell types are involved, and whether its effects are only mediated through reactive oxygen species (ROS).

Methods and Results—MtDNA damage occurred early in the vessel wall in Apolipoprotein E null (ApoE<sup>-/-</sup>) mice, before significant atherosclerosis developed. MtDNA defects were also identified in circulating monocytes and liver, and associated with mitochondrial dysfunction. To determine whether mtDNA damage directly promotes atherosclerosis, we studied ApoE<sup>-/-</sup> mice deficient for mitochondrial polymerase-γ proofreading activity (polG<sup>-/-</sup>/ApoE<sup>-/-</sup>). polG<sup>-/-</sup>/ApoE<sup>-/-</sup> mice showed extensive mtDNA damage and defects in oxidative phosphorylation, but no increase in ROS. polG<sup>-/-</sup>/ApoE<sup>-/-</sup> mice showed increased atherosclerosis, associated with impaired proliferation and apoptosis of vascular smooth muscle cells, and hyperlipidemia. Transplantation with polG<sup>-/-</sup>/ApoE<sup>-/-</sup> bone marrow increased features of plaque vulnerability, and polG<sup>-/-</sup>/ApoE<sup>-/-</sup> monocytes showed increased apoptosis and inflammatory cytokine release. To examine mtDNA damage in human atherosclerosis, we assessed mtDNA adducts in plaques, and in leukocytes from patients who had undergone virtual histology intravascular ultrasound characterization of coronary plaques. Human atherosclerotic plaques showed increased mtDNA damage compared with normal vessels; in contrast, leukocyte mtDNA damage was associated with higher-risk plaques but not plaque burden.

Conclusions—We show that mtDNA damage in vessel wall and circulating cells is widespread, causative and indicates higher risk in atherosclerosis. Protection against mtDNA damage and improvement of mitochondrial function are potential areas for new therapeutics.

Key words: animal model of human disease remodeling, atherosclerosis, pathophysiology
Mitochondria are often regarded as the cellular powerhouses through their ability to generate ATP, the universal fuel for metabolic processes. These protobacteria-related organelles are vital for eukaryotic function, yet they retain some independence, with their own genome and timing of replication. Mitochondria are also recognized as critical regulators of cell death and calcium signaling, and are the major cellular source of reactive oxygen species (ROS). Mitochondrial damage can therefore have a significant impact on cellular function, and has been implicated in the pathogenesis of both normal ageing and disease.

Human mitochondrial DNA (MtDNA) is a 16,569bp circular double-stranded molecule attached to the mitochondrial inner membrane. MtDNA encodes proteins of the electron transport chain (subunits of complexes I, III and IV and the ATP synthase (complex V)), including 13 essential oxidative phosphorylation genes, and ribosomal and transfer RNAs for protein synthesis. During mitochondrial respiration, electrons at complex I and III can react with molecular oxygen to form superoxide, although there may be multiple submitochondrial sites of reactive oxygen species (ROS) generation. MtDNA seems to be vulnerable to the accumulation of ROS-induced damage, including in vascular cells, perhaps because it lacks protective histones, and also because of its close proximity to the mitochondrial inner membrane. Errors made by the nuclear-encoded mtDNA polymerase-γ are another source of DNA defects, such as mutations and deletions. Once present, mtDNA damage persists longer than nuclear DNA damage, due to the lack of some specific DNA repair pathways that are present in the nucleus, particularly nucleotide excision repair. Mitochondrial damage is likely to lead to increased ROS production through disruption to oxidative phosphorylation.

MtDNA defects lead to mitochondrial dysfunction, promoting inflammation, apoptosis and cell senescence. These processes are pro-atherogenic, and indeed mtDNA damage is
associated with atherosclerosis. Human atherosclerotic aortas have increased mtDNA oxidative lesions compared with normal controls\textsuperscript{12}, and patients with coronary artery disease (CAD) have increased abundance of the “common” mtDNA 4977 bp deletion in the heart and circulating leukocytes\textsuperscript{13,14}. Furthermore, mtDNA damage is seen early in atherogenesis in mice deficient for Apolipoprotein E (ApoE\textsuperscript{−/−}), and impaired mitochondrial antioxidant activity can promote atherosclerosis\textsuperscript{12}. Although suggestive, it is not clear from these data whether mtDNA damage is a cause or consequence of tissue damage in atherosclerosis, and whether early mtDNA damage occurs only in the vessel wall. It is also unknown whether the observed mtDNA damage is sufficient to induce mitochondrial dysfunction, whether effects of mtDNA damage on atherosclerosis are mediated entirely by ROS, and whether mtDNA damage primarily promotes atherosclerosis or affects plaque composition.

We studied ApoE\textsuperscript{−/−} mice to confirm the early presence of mtDNA damage. To then determine whether mtDNA damage promotes atherosclerosis, we studied ApoE\textsuperscript{−/−} mice with deficient polymerase-γ proofreading activity (polG\textsuperscript{−/−}/ApoE\textsuperscript{−/−}). mtDNA damage promoted atherosclerosis and affected plaque composition independently of ROS in polG\textsuperscript{−/−}/ApoE\textsuperscript{−/−} mice, through effects on VSMCs, monocytes and hyperlipidemia. Importantly, human atherosclerotic plaques showed increased mtDNA damage, whilst leukocyte mtDNA damage was associated with higher-risk plaques but not plaque burden. Overall our results show that mtDNA damage is present, causative and indicates higher risk in atherosclerosis, with effects that can occur independently of ROS.

**Methods**

**Mice**

All animal experiments were performed under United Kingdom Home Office licensing, and
conformed to animal ethical committee approval. Both male and female C57Bl6/J ApoE+/− mice (Jackson Laboratory, Bar Harbor, Maine) were fed high fat (21% fat, Special Diet Services # 829100) or normal chow diet (3.4% fat, Special Diet Services # 801730) for 0, 7 or 14 weeks. Mice deficient for both polymerase-γ proofreading activity and ApoE (polG+/−/ApoE−/−) were generated by crossing C57BL6 PolG+/− (from Dr Nils Larsson) with C57BL6 ApoE−/− mice. Double heterozygotes were then backcrossed with ApoE−/− mice to generate polG+/−/ApoE−/− breeders. Breeding of these created both control (polG+/−/ApoE−/−) and experimental (polG−/−/ApoE−/−) mice. Genotyping of the mice was as described online. Both male and female littermate control (polG+/−/ApoE−/−) and experimental (polG−/−/ApoE−/−) mice were fat fed from 6-20 weeks. Feeding, bone marrow transplantation, lipid analysis, glucose and insulin tolerance testing and blood pressure measures were as previously described15,16.

**Body composition analysis**

To assess body fat and lean mass composition, experimental mice were scanned in a Minispec TDNMR analyzer (LF50, Bruker Optics, Massachusetts, USA) at 17 weeks of age.

**In vivo measurement of oxygen consumption and physical activity**

Oxygen consumption and activity assessment was as described Online.

**In vivo mitochondrial peroxide assessment**

MitoB, a mitochondria-targeted ratiometric probe, was used to assess in vivo mitochondrial H2O2 as previously described17.

**Histological analysis**

Analysis of plaque morphometry and composition was performed as described previously18.

**Quantitative PCR**

MtDNA damage was assessed as described previously12 (and Online). The 4977bp mutation was
assayed as described previously\textsuperscript{18}.

**Mitochondrial extraction and activity**

Mitochondria were extracted from liver tissue and assessed for complex I and citrate synthase activities as described previously\textsuperscript{16}.

**Fluorescent quantitative western blotting**

Western blotting was as described previously\textsuperscript{16}. Primary antibodies included citrate synthase (1:500)(ab96600, Abcam, UK) and tubulin (1:500)(2148s, Cell Signaling Technology, USA).

**Aortic respiration**

Respirometry was performed in permeabilised aortas, as described for other tissues\textsuperscript{19} and Online.

**Cell isolation and culture**

Leukocytes were pelleted from whole blood after erythrocyte lysis, and separated into CD11b\textsuperscript{+} monocytes by MACS separation (Miltenyi Biotec, Germany). Isolation purity was determined by Hoescht 33342 staining and CD11b immunocytochemistry of cytospins. Vascular smooth muscle cells (VSMCs) were cultured as previously described\textsuperscript{15}.

**Cellular ATP content and ROS assays**

Cellular ATP content and ROS were determined as described previously\textsuperscript{16} and Online.

**Cytokine Assays**

Monocytes were incubated with 1 µg/ml lipopolysaccharide (LPS) for 16 hours. Culture supernatants were analysed for TNF\textalpha, IL-1\textbeta, MCP1 and IL-6 as previously described\textsuperscript{20}.

**Time lapse videomicroscopy**

Cells were seeded into 24-well plates in medium containing 10% fetal bovine serum at 37°C, and incubated with 50 µM tert-butyl hydroperoxide (t-BHP) for 30 minutes. Cell proliferation and apoptosis were determined as described\textsuperscript{15}.

\textsuperscript{15}.
VIVA study

Patients with coronary artery disease were recruited from the VIVA (Virtual Histology in Vulnerable Atherosclerosis) study. DNA samples for mitochondrial lesions and serum 8-oxo-G were compared against VH-IVUS-defined plaque classification.

Statistical analysis

Data were tested for a normal distribution by examination of the histogram and normal probability plot, and by using the Shapiro-Wilk test. We used Student’s t test for pair-wise comparison or ANOVA with Bonferroni-Holm post hoc analysis for multiple comparisons for data following a normal distribution. Mann Whitney U test or Kruskal-Wallis test were used for non-parametric data. In conjunction with the Kruskal-Wallis test, Mann Whitney tests adjusted for multiple comparisons with the Bonferroni-Holm procedure were performed where indicated. Clinical and VH-IVUS parameters were assessed for association with mtDNA adducts using univariable and multivariable linear regressions. Each parameter was tested for an association with mtDNA adducts using univariable linear regression. Parameters with $p < 0.1$ on univariable analysis were entered into multivariable logistic regression model analysis, with $p < 0.05$ considered significant. Data are presented as means (standard deviation) for parametric data, medians (1st and 3rd quartiles (Q1-Q3)) for non-Gaussian data and frequency (percentage) for categorical data.

Results

Mitochondrial DNA damage occurs early in atherogenesis

Previous studies have shown that aortas of ApoE$^{-/-}$ mice develop mtDNA damage early in atherogenesis. However, it is unclear whether such lesions only occur in vessels or whether
they are also present in other cells or organs associated with atherosclerosis or lipid deposition. Whether the observed mtDNA damage results in mitochondrial dysfunction is also unknown. ApoE⁻/⁻ mice were fed either chow or high fat diet with plaque development analyzed at 0, 7 and 14 weeks, time points when chow-fed mice had either no significant atherosclerosis, or established plaques respectively (Figure 1A, Supplemental Figure 1). Baseline serum cholesterol was 6.6±0.23 mmol/l but increased with high fat feeding (Supplemental Figure 2).

We used qPCR analysis for mtDNA adducts to assess mtDNA damage. Oxidised bases, single and double strand breaks and abasic sites can accumulate through oxidative damage, and stop polymerase progression during PCR. Global damage can be assessed by comparing the amplification of a large segment of mtDNA with amplification of a short target, to control for mtDNA copy number12. Aortic mtDNA adducts increased in both groups, but were increased in chow-fed mice at 7 weeks (Figure 1B), even when no significant atherosclerosis was present. We also assessed mtDNA damage in peripheral monocytes and the liver. In these tissues, mtDNA adducts were significantly increased after 7 weeks of high fat diet, and by 14 weeks with both diets (Figure 1C-D). We examined respiratory complex subunit expression and complex activity to assess the functional consequences of mtDNA damage. There were similar levels of complex I, II, V subunit expression (normalized to the nuclear-encoded mitochondrial protein MnSOD) for all time points and both diets (Figure 1E, Supplemental Figure 3). In contrast, Complex I activity (normalized to the nuclear-encoded protein citrate synthase, a recognised marker of mitochondrial mass22) was reduced after 14 weeks of chow feeding, and by 7 weeks of high fat diet, correlating with the presence of significant atherosclerosis (Figure 1F). polG⁻/⁻/ApoE⁻/⁻ mice show increased mitochondrial DNA damage, no change in ROS, and reduced expression and respiration of mitochondrial complexes
We used mice doubly deficient for polymerase-γ proofreading activity and ApoE (polG−/−/ApoE−/−) to determine whether mtDNA defects promote atherosclerosis. polG−/− mice have a defect in exonuclease activity of a nuclear encoded mtDNA polymerase and accumulate widespread mtDNA point mutations and deleted mtDNA.23 mtDNA damage is associated with reduced mitochondrial respiratory complex activity and ATP production, despite a slight compensatory increase in mitochondrial mass23. In other studies polG−/− mice show premature aging without elevated mitochondrial oxidative damage23,24.

We first characterized the mtDNA damage and dysfunction in polG−/−/ApoE−/− mice and their polG+/+/ApoE−/− littermates. QPCR showed increased mtDNA adducts in polG−/−/ApoE−/− aortas and monocytes (Figure 2A-B). Although polG−/− mice have been reported to have normal ROS levels24,25, ApoE−/− mice have increased ROS, and it was important to determine ROS in polG−/−/ApoE−/− mice. In vivo mitochondrial hydrogen peroxide was assayed using the targeted probe MitoB17. MitoB accumulates within mitochondria where it reacts with H2O2 to form mitoP. Quantification of the mitoP/mitoB ratio enables measurement of mitochondrial H2O2. There were no significant differences in mitoP/mitoB ratio in aortic, heart and liver extracts between polG−/−/ApoE−/− and polG+/+/ApoE−/− mice (Figure 2C).

polG−/−/ApoE−/− aortas had reduced complex I and complex IV subunit expression, but there were no significant differences in the abundance of complexes II, III and citrate synthase (Supplemental Figure 4 and Figure 2D). We performed respirometry on aortic tissue to identify functional consequences of mtDNA damage and reduced complex expression. The aortas were permeabilized to allow substrate entry, and the respiration of different complexes assessed by using a specific sequence of substrates and inhibitors19. polG−/−/ApoE−/− aortas had reduced complex I- and complex IV-supported respiration compared with polG+/+/ApoE−/− control.
aortas (Figure 2E and 2G). No difference was observed in complex II-supported respiration (Figure 2F).

**polG−/−/ApoE−/− mice have increased atherosclerosis**

To determine the effect of mtDNA and dysfunction on atherosclerosis, polG−/−/ApoE−/− and their polG+/*/ApoE−/− littermates were fat fed from 6-20 weeks and atherosclerosis examined in three different vascular beds. Atherosclerosis in the aorta, brachiocephalic artery and descending aorta was increased in polG−/−/ApoE−/− mice compared with controls (Figure 3A-B). Whilst there was no difference in overall plaque composition, the percentage of cells undergoing apoptosis was increased, and percentage of proliferating cells decreased in polG−/−/ApoE−/− plaques (Table 1).

We have demonstrated that mtDNA damage occurs in both vessel wall and circulating monocytes in ApoE−/− mice, both of which might promote atherosclerosis. To determine the relative contribution of vessel wall and circulating bone marrow-derived cells, we transplanted ApoE−/− mice with polG−/−/ApoE−/− or control polG+/*/ApoE−/− marrow and fat fed mice from 6-20 weeks. Transplantation with polG−/−/ApoE−/− marrow did not affect extent of atherosclerosis in any vascular bed (Figure 3C-D). However, polG−/−/ApoE−/− marrow transplant increased the necrotic core area, and decreased the relative fibrous cap area, features of increased plaque vulnerability26 (Figure 3E).

**Effects of reduced polG proofreading activity on VSMCs and monocytes**

Our data suggest that the increased atherosclerosis seen in polG−/−/ApoE−/− mice, and changes in plaque composition seen after polG−/−/ApoE−/− transplant, represent changes in vessel wall cells and monocytes. We therefore examined the *in vitro* characteristics of cells derived from polG−/−/ApoE−/− mice. We initially assessed *in vitro* ROS with dichlorodihydrofluorescein diacetate (DCFDA), which fluoresces upon reaction with different oxidative species, including hydrogen
peroxide (H2O2), peroxynitrite and hydroxyl radicals. Although DCFDA is not specific for mitochondrial ROS, there was no difference between polG−/−/ApoE−/− and polG+/+/ApoE−/− VSMCs (Supplemental Figure 5), consistent with the in vivo MitoP/B data. In contrast ATP content assessed using a luciferin-luciferase assay was significantly reduced in polG−/−/ApoE−/− VSMCs and monocytes (Figures 4A-B).

To assess whether polG−/− cells have defects that might promote atherosclerosis, we used time-lapse videomicroscopy to examine cell proliferation and apoptosis. polG−/−/ApoE−/− VSMCs had markedly reduced cell proliferation, both basally and after treatment with the ROS-generating agent tert-butylhydroperoxide (Figure 4C). Indeed, under basal conditions polG−/−/ApoE−/− VSMCs proliferated poorly and senesced early (passage 7±2 vs. passage 19±2, mean±SEM). Both polG−/−/ApoE−/− VSMCs and monocytes showed increased apoptosis compared with polG+/+/ApoE−/− controls (Figures 4D-E). To assess whether mtDNA defects could promote an inflammatory phenotype, we examined LPS-induced monocyte cytokine release. polG−/−/ApoE−/− monocytes had increased release of IL1β and TNFα (Figure 4F), suggesting that increased pro-inflammatory cytokine release could contribute to the plaque vulnerability observed with polG−/− bone marrow transplantation.

**polG−/−/ApoE−/− mice show reduced adiposity, activity, and VO₂ but have hyperlipidemia**

Atherosclerosis is promoted not only by local effects on cells comprising the plaque, but also by systemic metabolic effects, including increased serum cholesterol, hypertension, and diabetes. The deficiency of DNA polymerase-γ activity in polG−/−/ApoE−/− mice was systemic; we therefore examined polG−/−/ApoE−/− mice for metabolic changes using a combination of dynamic testing and a comprehensive laboratory animal monitoring system (CLAMS). After 14 weeks of high fat feeding, female and male polG−/−/ApoE−/− mice had reduced total body weight compared with
control polG+/+ApoE+/+ mice (Figure 5A). Time domain nuclear magnetic resonance imaging showed that this was due to reduced percentage body fat (Figure 5B), which was confirmed on organ weight analysis. Both brown and white adipose tissue mass was reduced relative to total body weight, with no changes in other organs (Figure 5C). The CLAMS system was used to assay food and water consumption, physical activity and oxygen consumption. Although there were no differences in food and water consumption (data not shown), both physical activity and oxygen consumption (VO$_2$) were reduced in polG−/−/ApoE−/− mice (Figures 5D–E).

The alterations in body adiposity we observed (reduced weight, reduced fat %) can be associated with altered serum lipid levels and insulin sensitivity$^{27}$. Surprisingly, although there were no significant differences in serum lipids before and at 7 weeks of fat feeding (data not shown), polG−/−/ApoE−/− mice had increased serum total and LDL cholesterol after 12 weeks compared with polG+/+/ApoE+/+ mice (Figure 5F) – this was not seen in ApoE−/− mice transplanted with polG+/+/ApoE+/+ marrow (Supplemental Figure 6). In contrast, there were no significant differences in glucose or insulin tolerance testing (Figures 5G–H) or blood pressure (Supplemental Figure 7). We also examined whether the observed increased atherosclerosis in polG+/+ApoE+/+ mice was due to increased inflammatory cell indices. In contrast, we found a reduction in total leukocyte count with reduced lymphocyte fraction in both polG−/−/ApoE−/− mice and mice receiving polG+/−/ApoE−/− mouse bone marrow, and also reduced monocyte count in polG−/−/ApoE−/− mice (Supplemental Figure 8).

**Human plaques show increased mtDNA damage and mtDNA damage is associated with higher-risk plaques**

Our data show that mtDNA defects are present, widespread, and reduce mitochondrial function in ApoE−/− mice and promote atherogenesis in polG+/+ApoE+/+ mice; however, the relevance of
these findings to human disease is unclear. In particular, the bone marrow transplantation data suggest that circulating cells with mtDNA damage could promote plaque vulnerability, potentially via increased local cytokine release. We therefore examined mtDNA adducts and the 4977bp mitochondrial deletion in normal human ascending aorta and age and sex-matched carotid endarterectomy plaques. Human plaques showed a 2-fold increase in mtDNA adducts and a 1.7-fold increase in the 4977bp deletion, compared with normal vessels (Figure 6A-B).

To examine whether leukocyte mtDNA damage correlates with atherosclerosis extent or plaque vulnerability in humans, we assessed mtDNA adducts in patients from the Virtual Histology in Vulnerable Atherosclerosis (VIVA) trial. VIVA used 3-vessel virtual histology intravascular ultrasound (VH-IVUS) to examine 1,096 plaques in 170 patients undergoing intracoronary stenting for stable (n=100) or acute coronary syndromes (ACS) (n=70). VH-IVUS can classify plaques by their composition as different plaque components may be identified by their backscatter spectrum (Figure 6C), as well as accurately measure plaque burden. VIVA, and the recent PROSPECT study, showed that VH-IVUS-defined thin-capped fibroatheromas (VHTCFA) have the highest risk of cardiovascular events on subsequent follow up. We examined the association between patient demographics, VH-IVUS-defined plaque classification or plaque burden with mtDNA adducts.

On multivariable analysis, mtDNA adducts were not associated with patient demographics including family history or previous history of MI, smoking, age, sex, hypertension or drug therapy, although were positively associated with diabetes (sB=0.33, p=0.012), and negatively with serum cholesterol (Table 2). mtDNA adducts did not change significantly 3m after PCI in stable angina patients, but were reduced after treatment in ACS patients (Supplemental Figure 9). Whilst there was an association between serum 8-oxo-G, a
DNA marker of oxidation, and mtDNA adducts on univariable analysis, this was not statistically significant on multivariable analysis (Table 2). In contrast, whilst there was no association between leukocyte mtDNA adducts and 3-vessel or culprit vessel plaque volume, 3-vessel and culprit vessel VHTCFA number were strongly associated with both mtDNA adducts (Table 2) and the 4977 mtDNA deletion (Supplemental Table 1). Importantly, other VH-identified plaque types were not positively associated with mtDNA adducts, and VHThCFA (thick cap fibroatheromata) were negatively associated with the 4977 mtDNA deletion.

**Discussion**

MtDNA damage is implicated in a variety of ageing-related phenotypes\(^{23,25}\) including atherosclerosis\(^{13}\). MtDNA defects lead to impaired mitochondrial function, resulting in increased ROS, which in turn damage mitochondria further. Oxidative stress is seen in atherosclerosis\(^{29}\), and ROS are seen as a major effector pathway by which mtDNA damage might promote ageing and age-related disease\(^{30}\). In contrast, mtDNA damage can also directly promote inflammation, apoptosis and senescence\(^{8,9}\), all processes involved in both atherogenesis and the transition to an unstable plaque phenotype. Furthermore, plaques comprise cells from both the vessel wall and the bone marrow, and monocyte invasion of the vessel wall is one of the earliest events in atherosclerosis. It is therefore unclear whether the vessel wall is the only target of mtDNA damage in atherosclerosis, and whether the effect is only mediated through ROS.

In ApoE\(^{-/-}\) mice, we show that mtDNA damage occurs in arteries, circulating cells and other organs during atherogenesis. Moreover, we confirm that mtDNA damage is an early event in atherogenesis\(^{12}\), detectable even before significant atherosclerotic plaque has developed. mtDNA damage generally increases with time in these mice, is increased by a high fat diet, and
is sufficient to reduce mitochondrial function, as assayed by complex I activity. This early onset raises the possibility that mtDNA damage has a causative role, rather than being just a late consequence of extensive ROS and tissue damage in advanced plaques. We therefore studied mice defective for both proofreading activity of mtDNA polymerase-γ and ApoE. We show that mtDNA damage directly promotes atherogenesis, in part through effects on VSMCs and through increasing serum lipids. Transplant of polG−/−/ApoE−/− bone marrow reduced the relative fibrous cap area and increased the necrotic core area of plaques, consistent with a more unstable phenotype.

Although the mechanisms inducing mtDNA damage in fat-fed ApoE−/− mice and polG−/− mice are different, the lesions caused have many similarities and we show that the downstream consequences on complex activity are similar. polG−/− mice accumulate mtDNA mutations in an approximately linear manner24, as the polymerase defect introduces point mutations and deletions into the mitochondrial genome23,25. Although the qPCR adducts assay detects a variety of lesions, including strand breaks and abasic sites12, these lesions can result from base excision repair of mutations31. polG−/−/ApoE−/− mice showed increased mtDNA adducts, and reduced expression of complexes I and IV, which may be especially vulnerable to mtDNA damage because they have the highest number of mtDNA-encoded subunits32. Although the exact mechanisms underlying the decreased abundance are unclear, reduced transcript levels may be involved33. The changes in complex expression in polG−/−/ApoE−/− mice led to reduced complex I and IV respiration and reduced cellular ATP content. Whilst not directly examined in our study, reduced complex activity could also contribute to the decreased respiration23.

Oxidative stress is considered to contribute towards atherogenesis29, and ROS have been suggested to play a key role in the mitochondrial theory of ageing30. However, despite extensive
point mutations and deletions of mtDNA, polG−/− mice do not demonstrate increased general levels of mitochondrial ROS in bulk tissues24,25. In addition, levels of antioxidant enzymes, protein carbonylation, and aconitase activity measurements indicate no or only minor oxidative stress in polG−/− mouse tissues24, although reduction in mitochondrial ROS can alleviate some of the features in polG−/− mice34. We assessed ROS with the general probe DCFDA in cultured cells and the targeted probe MitoB in vivo17. polG−/−/ApoE−/− mice or cells showed no increase in ROS, suggesting for the first time that mtDNA defects can promote atherosclerosis independently of ROS. We might anticipate that complex dysfunction would lead to increased oxidative stress in polG/ApoE−/− mice. However, the decreased abundance of ROS-generating complexes and low mitochondrial membrane potential noted by others33 in polG−/− mice might explain why no increase was seen.

Despite no increase in ROS, polG−/−/ApoE−/− VSMCs and monocytes showed increased apoptosis and decreased proliferation, and plaques in polG−/−/ApoE−/− mice showed a similar phenotype. Both VSMC and monocyte/macrophage apoptosis have been reported to promote atherosclerosis and can result from mitochondrial dysfunction10,11,35. Mitochondria are important in regulating apoptosis through the mitochondrial permeability transition pore (MPTP)32. Opening of the MPTP leads to release of cell death-promoting factors, such as cytochrome C and apoptosis-inducing factor36,37. Decreased levels of ATP favor pore opening38, and therefore increase apoptosis. Decreased ATP also promotes cell senescence through AMP-activated protein kinase (AMPK), which decreases the expression of proliferative genes, such as cyclin A and B39-41.

We also show that MtDNA defects may increase atherosclerosis through systemic effects. polG−/−/ApoE−/− mice showed reduced total body weight, with reduced fat mass and increased
serum lipids. This picture is suggestive of lipodystrophy, a condition for which mitochondrial dysfunction has been implicated. In lipodystrophy, adipocyte stores are compromised by impaired fatty acid oxidation, decreased adipogenesis and increased adipocyte apoptosis; the subsequent fat redistribution results in hyperlipidemia.

Contrasting with our findings in polG−/−/ApoE−/− mice, transplantation with polG−/−/ApoE−/− bone marrow did not alter atherosclerosis extent, but increased necrotic core and decreased relative fibrous cap areas, features of increased plaque vulnerability. These effects may be due to increased mtDNA damage and subsequent cell death, as polG−/−/ApoE−/− monocytes showed reduced ATP content and increased apoptosis. Increased macrophage apoptosis promotes necrotic core expansion as efferocytosis is impaired and overwhelmed, leading to secondary necrosis and inflammation. polG−/−/ApoE−/− monocytes also had increased secretion of TNFα and IL-1β. The pro-inflammatory phenotype may result from increased MPTP opening, which leads to mtDNA release into the cytosol. mtDNA binds to and activates the inflammasome, with subsequent increased expression and release of IL1β and TNFα.

Having shown that mtDNA defects promote atherosclerosis and plaque vulnerability in a murine model, we examined the relevance of these findings in human disease. Consistent with previous work, we show that human plaques have increased mtDNA damage compared with age- and sex-matched normal aortas. More importantly, we show that leukocyte mtDNA damage is associated with higher risk plaques as assayed by virtual histology intravascular ultrasound (VH-IVUS). The recent VIVA and PROSPECT studies showed that VH-IVUS-defined thin-capped fibroatheromas (VHTCFA) have the highest risk of cardiovascular events on subsequent follow up. Consistent with our findings that monocyte mtDNA damage promotes features of plaque vulnerability, leukocyte mtDNA damage does not correlate with plaque volume in
humans, and was associated only with VH-TCFA. The association between mtDNA adducts and 8-oxo-G was consistent with previous findings that ROS can induce mtDNA damage; however, the correlation was not present on multivariable analysis, and the absence of an association between 8-oxo-G and the 4977 bp deletion suggests that mtDNA defects can occur independently of ROS.

Although ROS are undoubtedly important in mtDNA damage and atherosclerosis, our data suggest another mechanism linking mtDNA defects and atherosclerosis. MtDNA defects lead to decreased expression of respiratory complexes and reduced mitochondrial respiration in VSMCs, monocyte/macrophages and other organs. ATP content is reduced, which promotes apoptosis and inhibits cell proliferation. These processes also compromise adipose tissue stores promoting hyperlipidemia, which, together with VSMC apoptosis, increases atherosclerosis. MtDNA defects also promote plaque vulnerability through increased monocyte apoptosis and inflammatory cytokine release.

In summary, we show that mtDNA damage can promote atherosclerosis and plaque vulnerability, and indicates increased risk in patients. Protection against mtDNA damage, and improvement of mitochondrial function, are potential areas for new therapeutics. Biomarkers of mtDNA damage also show promise to risk-stratify CAD patients.

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**Conflict of Interest Disclosures:** None.
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Table 1. Apoptosis is increased and cell proliferation reduced in polG<sup>-/-</sup>/ApoE<sup>-/-</sup> mice plaques.

<table>
<thead>
<tr>
<th></th>
<th>SMA positive area (%)</th>
<th>MAC 3 positive area (%)</th>
<th>Necrotic core area (%)</th>
<th>Cap area / Plaque area</th>
<th>TUNEL positive cells (%)</th>
<th>Ki67 positive cells (%)</th>
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<td>Control</td>
<td>25.52 ± 2.41</td>
<td>55.71 ± 5.08</td>
<td>69.15±1.8</td>
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<td>0.887 [0.096-1.24]</td>
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<td>polG&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>24.78 ± 2.19</td>
<td>58.42 ± 4.35</td>
<td>71.48±2.36</td>
<td>22.1±1.5</td>
<td>1.55 [0.82-2.61] *</td>
<td>1.85 ± 0.2*</td>
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Percentage α-smooth muscle cell actin (SMA), Mac-3, necrotic core and fibrous cap/plaque areas, and percentage of cells undergoing apoptosis (TUNEL) or proliferation (Ki67) in control polG<sup>+/+</sup>/ApoE<sup>-/-</sup> and polG<sup>-/-</sup>/ApoE<sup>-/-</sup> plaques. Data are means ± SEM for parametric data, and medians [Q1-Q3] for non-parametric data. *p<0.05, n=12.

Table 2. MtDNA adducts are associated with higher risk plaque

<table>
<thead>
<tr>
<th>Clinical Parameters</th>
<th>Mean (sd), median [Q1-Q3]</th>
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<th>Multivariable analysis</th>
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<td></td>
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<td>Beta coefficient</td>
<td>P value</td>
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<td>Age (years)</td>
<td>62.3 (10.4)</td>
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<td>Male</td>
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IVUS Parameters

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Sd=standard deviation, Q1-Q3=first and third quartiles, vol=volume, ACS= acute coronary syndrome
MI=myocardial infarction, VHPIT=VH-IVUS-identified pathological intimal thickening, VHFCa=VH-IVUS-identified fibro-calcific plaque, VHThCFA=VH-IVUS-identified thick-capped fibroatheroma.
Figure Legends:

Figure 1. MtDNA damage occurs early in atherogenesis and induces mitochondrial dysfunction. (A). Quantification of aortic root plaque areas in ApoE^{-/-} mice fed chow or high fat diet for 0, 7 or 14 weeks (n=4-6 per group). (B-D). mtDNA adducts in aortic arch (B), monocytes (C), or liver (D). (E). Quantification of complex protein expression normalized to the nuclear-encoded protein MnSOD. (F). Liver mitochondria complex I activity, normalized to the nuclear-encoded protein citrate synthase. Bars represent medians (A-B); other data are means+SEMs.

Figure 2. polG^{-/-}/ApoE^{-/-} mice show increased mitochondrial DNA damage, and reduced expression and respiration of mitochondrial complexes. (A-B). MtDNA adducts in aortas (A) or monocytes (B) in polG^{-/-}/ApoE^{-/-} or control polG^{+/-}/ApoE^{-/-} mice (n=12). (C). MitoP/B ratio in aorta, heart and liver of polG^{-/-}/ApoE^{-/-} and control polG^{+/-}/ApoE^{-/-} mice. (D). Normalized protein expression for respiratory complexes in mouse aortas (n=5). (E-G). Complex I (E), II (F), or IV (G) supported respiration of aortas from polG^{-/-}/ApoE^{-/-} and control polG^{+/-}/ApoE^{-/-} mice, (n=5). Bars represent medians (A) and (C); other data are means+SEMs.

Figure 3. polG^{-/-}/ApoE^{-/-} mice have increased atherosclerosis. (A). Aortic root sections stained with haematoxylin and eosin, α-SMA or Mac 3 in polG^{-/-}/ApoE^{-/-} or control polG^{+/-}/ApoE^{-/-} mice fat fed from 6-20 weeks of age. (B). Quantification of plaque area in aortic root, brachiocephalic artery and descending aorta in polG^{-/-}/ApoE^{-/-} and control polG^{+/-}/ApoE^{-/-} mice. (C). Aortic root sections stained with H+E in ApoE^{-/-} mice transplanted either with polG^{+/-}/ApoE^{-/-} or polG^{-/-}/ApoE^{-/-} marrow and fat fed for 14 weeks. (D-E). Quantification of plaque area (D) or necrotic...
core and fibrous cap/plaque areas (E) in transplanted mice. Bars represent medians. n=12 mice.

Scale bars (A,C) represent 500 μm.

**Figure 4.** polG+/ApoE−/− VSMCs have reduced ATP, reduced proliferation and increased apoptosis. polG+/ApoE−/− monocytes have increased inflammatory cytokine release. (A-B). ATP content in VSMCs (n=5)(A), or monocytes (n=5)(B), from polG+/ApoE−/− and control polG+/+/ApoE−/− mice. (C-D). Percentage of VSMCs undergoing proliferation (C) or apoptosis (D) basally or after t-BHP treatment (n=6). (E-F). Apoptosis (E) and LPS-induced cytokine secretion (F) in monocytes derived from polG+/ApoE−/− or control ApoE−/− mice (n=5). Bars represent medians (C); other data are means+SEM.s.

**Figure 5.** polG+/ApoE−/− mice show reduced adiposity, activity, and VO2 but have hyperlipidemia. (A). Total body weight for polG+/ApoE−/− and control polG+/+/ApoE−/− mice after 14 weeks of fat feeding (n=12). (B). % body fat in polG+/ApoE−/− mice and control polG+/+/ApoE−/− mice (n=12) assessed by time-domain nuclear magnetic resonance imaging. (C). Organ weight relative to total body weight for same groups as in (A). (D). Activity measured as beam breaks in polG+/ApoE−/− and control polG+/+/ApoE−/− mice (n=3). (E). Oxygen consumption (VO2) normalized to body mass for same groups as in (D). (F). Serum lipids in polG+/ApoE−/− and control polG+/+/ApoE−/− mice after 12 weeks of fat feeding (n=12). (G). Fasted glucose tolerance test in polG+/ApoE−/− and control polG+/+/ApoE−/− mice after 9 weeks of fat feeding (n=12). (H). Insulin tolerance test, for same groups as (G). BAT=brown adipose tissue, WAT=white adipose tissue. Chol=serum cholesterol, Trig=serum triglyceride. Bars represent medians (F); other data are means+SEM.s.
Figure 6. Human plaques show increased mtDNA damage and deletion and mtDNA damage is associated with higher-risk plaques. (A-B). Expression of mtDNA adducts (A) or the 4977bp mtDNA deletion (B) in human normal aorta or atherosclerotic plaque (n=9). (C). VH-IVUS plaque classification as used in VIVA. Plaque composition is coded using different colors: Dark green=fibrous tissue, yellow =fibrofatty tissue, red=necrotic core, white=calcification. Data are means+SEMs.
Figure 1
Figure 2
Figure 3
**Figure 4**

- **Panel A**: Comparison of nmoles/10^5 cells between Control and polG^-/-.
  - p = 0.02

- **Panel B**: Comparison of nmoles/10^6 cells between Control and polG^-/-.
  - p = 0.02

- **Panel C**: Cumulative proliferation/24 hours (%).
  - Basal
  - t-BHP
  - p < 0.001

- **Panel D**: Cumulative apoptosis/24 hours (%).
  - Basal
  - t-BHP
  - p = 0.003
  - p = 0.008

- **Panel E**: Cumulative % Apoptosis/24 hours.
  - Control
  - polG^-/-
  - p = 0.046

- **Panel F**: Levels of cytokines (pg/ml).
  - IL-6
  - IL-1β
  - TNF-α
  - MCP-1
  - p = 0.013
  - p = 0.208
  - p = 0.15
  - p = 0.03
Figure 5
Figure 6

Adaptive intimal thickening (VHAIT)

Pathological intimal thickening (VHPIT)

Fibrocalcific plaque (VHFCa)

Thick cap fibroatheroma (VHThCFA)

Thin cap fibroatheroma (VHTCFA)

Lesions / 10kb

Relative expression

Aorta Plaque

p = 0.047

p = 0.038
Mitochondrial DNA Damage can Promote Atherosclerosis Independently of Reactive Oxygen Species through Effects on Smooth Muscle Cells and Monocytes, and Correlates with Higher Risk Plaques in Humans

Emma Yu, Patrick A. Calvert, John R. Mercer, James Harrison, Lauren Baker, Nichola L. Figg, Sheetal Kumar, Julie J. Wang, Liam A. Hurst, Daniel R. Obaid, Angela Logan, Nick E. J. West, Murray C. H. Clarke, Antonio Vidal-Puig, Michael P. Murphy and Martin R. Bennett

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SUPPLEMENTAL MATERIAL

Supplemental methods

Mice genotyping

Genotyping of ApoE−/− mice was performed by PCR using company protocols (Jackson Laboratory). The genotyping of polG−/− mice was performed by PCR using the following primers at 5µM: FWD: CTTCGGAAGAGCAGTCGGGTG, REV: GGGCTGCAAAGACTCCGAAGG. The cycling parameters were 1 cycle of 94°C, 1 min and 30 cycles of 94°C, 30 s; 61°C, 30s; 72°C, 1min. Expected product sizes were polG+/+ 520 bp, polG+/− 520 and 720 bp, polG−/− 720 bp.

In vivo measurement of oxygen consumption and physical activity

After 13 weeks of high fat feeding, mice were transferred to individual monitoring cages, kept at 22 C, under an alternating 12h:12h light-dark cycle. Following 24 hours of acclimatization, mice were assessed for 48 hours. Water and food consumption, ambulatory activity, and respiratory exchange ratio (VCO2/VO2) were measured using a continuous laboratory animal monitoring system (CLAMS, Columbus Instruments, OH) fitted with an indirect calorimetry system (miniMOX; University of Cambridge, Cambridge, UK).

QPCR for mitochondrial DNA lesions

QPCR for murine mitochondrial DNA lesions was performed using previously described primers. For assessment of human mtDNA adducts, primers used at 5 µM were as follows:

Long primers: producing a 10235 bp product, FWD: ACATACCCATGGCCAACCT, REV: TATGTTTGC GGT TCGATGA. Short primers: producing a 113 bp product, FWD:ACATACCCATGGCCAACCT, REV: GGGCCTTTGC G TATGTTGTAT.
QPCR was performed in triplicate, on 20 ng of sample DNA in 20 µl reactions on a Rotor-Gene™ 6000 QPCR thermocycler (Corbett Research, AU). Fast Sybr Mix (Qiagen, Germany) was used for the short reaction and PFU Ultra II Hotstart PCR Master Mix (Agilent Technologies, CA, USA) with 1 in 400 dilution of Eva Green (Biotium, CA, USA) for the long reaction. Cycling parameters for the short reaction were 95°C for 5 minutes followed by 45 cycles of 94°C for 30s, 60°C for 30s. Conditions for the long amplification were 94°C for 2 minutes followed by 45 cycles of 92°C for 30s, 71°C for 30s, and 68°C for 5 minutes. The relative amplification of the 10kb product was calculated by the comparative Ct method\(^2\). DNA lesion frequencies were calculated using Poisson transformation\(^3\).

QPCR for the common 4977 bp mtDNA deletion was performed as previously described\(^1\). For human lesions, primers used at 5 µM were as follows: Control product: FWD: ACATACCCATGGCCAACCT, REV: GGGCCTTTGCGTAGTTGTAT, producing a 113 bp product. Mutant product primers: FWD: CTATTGATCCCCACCTCCAA, REV: GCGAGGGCTGTGAGTTTTAG, producing a 332 bp product. Reaction conditions were as described for the short adducts reaction.

**Cellular ATP content and ROS assays**

Cellular ATP content was determined with a luciferin-luciferase-based bioluminescence assay (ATP lite: Luminescence ATP detection system, Perkin Elmer, USA) as per the manufacturer’s instructions. Luminescence was measured on a Synergy HT Plate Reader (Biotek Instruments, Vermont, USA) with standard curves generated from known concentrations of ATP standards. Determination of
intracellular ROS with 2′,7′-dichlorodihydrofluorescein diacetate was performed as previously described\textsuperscript{16}.

**Aortic respiration**

To assess mitochondrial respiration, descending aortas were dissected and maintained in preservation solution (BIOPS: 10 mM Ca-EGTA buffer, 0.1 µM free calcium, 20 mM imidazole, 20 mM taurine, 50 mM MES, 0.5 mM dithiothreitol, 6.56 mM MgCl\textsubscript{2}, 5.77 mM ATP, 15 mM phosphocreatine, pH 7.1) at 4°C. Samples were then permeabilized in 50 µg/ml saponin solution before washing three times in respiration buffer (0.5 mM EGTA, 3 mM MgCl\textsubscript{2}·6H\textsubscript{2}O, 60 mM K-lactobionate, 20 mM taurine, 10 mM KH\textsubscript{2}PO\textsubscript{4}, 20 mM HEPES, 110 mM mannitol, 0.3 mM dithiothreitol, 1 mg/ml fatty acid free BSA, pH 7.1). Respirometry was performed at 37°C with a Clarke-type oxygen electrode (Strathkelvin Instruments Ltd, UK).

Complex I-supported respiration rates were acquired using 10 mM glutamate and 5 mM malate. State 3 respiration was stimulated by the addition of 5 mM ADP. After the addition of 1 µM rotenone, complex II-supported respiration was assessed with 10 mM succinate. 0.5 mM \textit{N,N,N',N'-}Tetramethyl-p-phenylenediamine (TMPD) and 2 mM ascorbate were then used to induce complex IV respiration, after inhibition of complex III with 5 µM antimycin. Outer mitochondrial membrane intactness was assessed by the addition of 10 µM cytochrome C. Tissues were removed from the electrode chambers and dried, with oxygen flux expressed as nanomoles O\textsubscript{2} per minute per dry weight.

**Serum 8-oxo-G assays**
Serum samples were filtered through a 0.45 µm filter and assessed for 8-oxo-G using a competitive ELISA assay (OxiSelect™ Oxidative DNA Damage ELISA #STA 320, Cell Biolabs Inc, California, USA) as per the manufacturer’s instructions. Absorbance was measured on a Synergy HT Plate Reader (Biotek Instruments, Vermont, USA) with standard curves generated from known concentrations of 8-oxo-G standards.
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<td>Age (years)</td>
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<td>122 (76.7%)</td>
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<td>ACS presentation</td>
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**Supplemental Table 1. Association between clinical and virtual histology intravascular ultrasound factors and mtDNA 4977 deletion**

sd (standard deviation), Q1-Q3 (first and third quartiles), Vol (volume), MI (myocardial infarction), LDL (low density lipoprotein), HDL (high density lipoprotein), VH-IVUS (virtual histology intravascular ultrasound), VHPIT (VH-IVUS identified pathological intimal thickening), VHFCa (VH-IVUS identified fibro-calcific plaque), VHThCFA VH-IVUS identified thick-capped fibroatheroma, VHTHCFA VH-IVUS identified thin-capped fibroatheroma, mtDNA (mitochondrial DNA).
Supplemental Figures

Supplemental Figure 1
Representative images of aortic root sections, stained with haematoxylin and eosin. Scale bars represent 500 µm.

Supplemental Figure 2
Serum cholesterol and triglycerides in ApoE<sup>−/−</sup> mice fed either chow or a high fat diet from 6w of age for 0, 7 or 14w.
Supplemental Figure 3
Quantitative fluorescent Western blot of aortic mitochondrial respiratory complex (CI-CIV) proteins in ApoE-/- mice fed chow or high fat diet for 0, 7 or 14w. The nuclear encoded manganese superoxide dismutase (MnSOD) was used as the control.

Supplemental Figure 4
Quantitative fluorescent Western blot of aortic mitochondrial respiratory complex (CI-CIV) proteins, and citrate synthase (CS) in control or polG^-/- mice. Tubulin (tub) was used as control.

Supplemental Figure 5
DCFDA fluorescence in VSMCs derived from polG^+/+ApoE^-/- (control)(n=3) or polG^-/-ApoE^-/- mice (n=4).
Supplementary Figure 6. Lipid levels in bone marrow transplanted mice
Serum lipids in ApoE−/− mice transplanted with polG−/−/ApoE−/− or control ApoE−/− marrow, after fat feeding from 6-20w. n=12. Chol=serum cholesterol, Trig=serum triglyceride

Supplemental Figure 7
Mean systolic blood pressure in polG+/+ /ApoE+ (control) or polG−/−/ApoE−/− mice undergoing fat feeding from 6-20w. n=12.
Supplemental Figure 8

(A) Blood counts in polG+/ApoE+ and control ApoE−/− mice after fat feeding from 6-20w. (B) Blood counts in ApoE−/− mice transplanted with polG+/ApoE−/− or control polG+/+ApoE−/− marrow and fat feeding from 6-20w. (n=12).
Supplemental Figure 9

(A-B) mtDNA adducts at baseline (0 months, pre-PCI) and 3m later in patients presenting with stable angina (A) or ACS (B).
References

