

Endothelial Cell Bioenergetics and Mitochondrial DNA Damage Differ in Humans Having African or West Eurasian Maternal Ancestry

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Background—We hypothesized that endothelial cells having distinct mitochondrial genetic backgrounds would show variation in mitochondrial function and oxidative stress markers concordant with known differential cardiovascular disease susceptibilities. To test this hypothesis, mitochondrial bioenergetics were determined in endothelial cells from healthy individuals with African versus European maternal ancestries.

Methods and Results—Bioenergetics and mitochondrial DNA (mtDNA) damage were assessed in single-donor human umbilical vein endothelial cells belonging to mtDNA haplogroups H and L, representing West Eurasian and African maternal ancestries, respectively. Human umbilical vein endothelial cells from haplogroup L used less oxygen for ATP production and had increased levels of mtDNA damage compared with those in haplogroup H. Differences in bioenergetic capacity were also observed in that human umbilical vein endothelial cells belonging to haplogroup L had decreased maximal bioenergetic capacities compared with haplogroup H. Analysis of peripheral blood mononuclear cells from age-matched healthy controls with West Eurasian or African maternal ancestries showed that haplogroups sharing an A to G mtDNA mutation at nucleotide pair 10398 had increased mtDNA damage compared with those lacking this mutation. Further study of angiographically proven patients with coronary artery disease and age-matched healthy controls revealed that mtDNA damage was associated with vascular function and remodeling and that age of disease onset was later in individuals from haplogroups lacking the A to G mutation at nucleotide pair 10398.

Conclusions—Differences in mitochondrial bioenergetics and mtDNA damage associated with maternal ancestry may contribute to endothelial dysfunction and vascular disease. (*Circ Cardiovasc Genet.* 2016;9:26-36. DOI: 10.1161/CIRCGENETICS.115.001308.)

Key Words: coronary artery disease ■ DNA, mitochondrial ■ endothelial cells ■ genetics
■ human umbilical vein endothelial cell ■ mitochondria

Cardiovascular disease (CVD) has been the leading cause of mortality and morbidity in the United States for the past century.¹ Although racial differences in the prevalence of CVD have been well documented, the mechanisms that underlie these differential susceptibilities have yet to be determined. Endothelial dysfunction is generally considered to be the initiating factor in CVD development, and increased cellular oxidant stress has been linked to endothelial dysfunction. In this regard, there have been multiple endogenous sources of reactive oxygen species (ROS) implicated in endothelial

dysfunction and CVD. Among them, the mitochondrion has been noted to be both a primary source and a target of oxidants.

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The mitochondrion is a multifunctional organelle, serving as the site for electron transport, oxidative phosphorylation, the citric acid cycle, β -oxidation, steroidogenesis, and many other important cellular functions, including growth, oxidant generation, and programmed cell death. Mitochondria also have their own genome (mitochondrial DNA [mtDNA])

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that encodes the translational machinery (2 rRNAs and 22 tRNAs) necessary for the generation of polypeptides from 13 mtDNA-encoded genes that are essential for electron transport and oxidative phosphorylation. During oxidative phosphorylation, mitochondria couple electron transport with proton translocation, which in turn enables the production of ATP. Mitochondrial ROS generation occurs when electrons leak from electron transport (estimated to account for $\approx 4\%$ to 5% of total oxygen consumption) resulting in the formation of superoxide ($O_2^{\cdot-}$).^{2,3} It has been proposed that variation in mitochondrial energy and oxidant production exists in normal cells that would translate into differences in mitochondrial oxygen utilization between individuals. Moreover, these changes are attributed to the known inherent genetic variability of the mtDNA, which characterize mtDNA haplogroups.⁴⁻⁶

Indeed, epidemiological studies have indicated that associations exist between certain mtDNA haplogroups and many age-related diseases, including ischemic CVD, hypertension, type-2 diabetes mellitus, obesity, metabolic syndrome, cancer, and neurodegenerative diseases.⁷⁻¹² Similarly, animal studies indicate that mtDNA haplotype can influence longevity, risk of type-2 diabetes mellitus, CVD, cancer, and fatty liver disease development.¹³⁻²¹ Although cellular studies using animal models of mtDNA variation or immortalized cybrid cell lines have yielded conflicting results in terms of mitochondrial function and ROS production,²²⁻²⁴ to our knowledge, no studies have compared mitochondrial function or integrity in normal cells from healthy individuals who represent different mtDNA haplogroups. In fact, all published data have used systems that are reliant on immortalized cell lines, thereby potentially confounding the interpretation of these data within the normal cellular context.

In this study, endothelial cells harboring either mitochondrial haplogroup H or L (representing West Eurasian or African mtDNAs, respectively) demonstrated distinct bioenergetic profiles. These profiles indicate that cells belonging to haplogroup L were more economical in terms of energy generation (used less oxygen for ATP production), but also had higher basal levels of mtDNA damage. MtDNA damage was also quantified from peripheral blood mononuclear cells (PBMCs) collected from age-matched healthy controls and compared between individuals belonging to West Eurasian and African haplogroups. Results show that mtDNA damage is significantly higher in groups belonging to African and West Eurasian maternal ancestries that share a single-nucleotide polymorphism (SNP) at nucleotide pair 10398. Furthermore, additional studies comparing patients with CVD and healthy, age-matched individuals found that mtDNA damage significantly correlated with vascular function and remodeling. These data demonstrate that (1) mitochondrial bioenergetics can vary in normal, primary cells having distinct mtDNA haplogroups, (2) mtDNA damage varies by mtDNA haplogroups, and (3) increased mtDNA damage segregates with both CVD and differences in vascular function and remodeling. Thus, our results provide new insights into the mechanisms that govern CVD susceptibility in humans.

Methods

Primary Endothelial Cells

Primary human umbilical vein endothelial cells (HUVECs) were chosen for these studies because of the desire to minimize the possibility of pre-existent factors influencing endothelial function (age, CVD risk factor exposure, etc) and obtain normal, naive endothelial cells. Single-donor HUVECs were obtained from individual cords from male infants (Lonza, Walkersville, MD), and the mtDNA haplotype was determined by direct sequencing as previously described.^{25,26} For these studies, HUVEC samples belonging to haplogroup H (West Eurasian, n=5) or L (African, n=9) were selected and maintained in a humidified incubator at 37°C with 5% CO₂ in EGM2 growth medium (Lonza, Walkersville, MD). All experiments used cells between passages 5 and 9. HUVECs within haplogroups H and L were used because their mtDNAs represent the most common maternal lineages in North American whites and blacks, respectively. All HUVEC studies were performed in triplicate.

Measurement of Mitochondrial Function and Estimation of Oxygen Utilization

A Seahorse Bioscience XF24 extracellular flux analyzer was used to measure mitochondrial function in intact HUVECs. The XF24 creates a transient, 7- μ L chamber in specialized microplates that allows for the determination of oxygen and proton concentrations in real time.^{27,28} Thus, the rates of oxygen consumption and proton production can be measured across several samples at a time. Measurements of extracellular flux were made in unbuffered media. For these experiments, cells were seeded at 30000 cells per well onto Seahorse Bioscience (North Billerica, MA) V7 tissue culture plates in culture media and allowed to adhere and grow for 24 hours. The following day, the media was changed 1 hour before the start of the extracellular flux assay to DMEM supplemented with 25 mmol/L D-glucose, 2 mmol/L L-glutamine, and 1 mmol/L pyruvate. The pH of the medium was adjusted to 7.4 with NaOH. To allow comparison between experiments, data are expressed as the rate of oxygen consumption in pmol/min per 3×10^4 cells. To assay mitochondrial function, oligomycin, carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone, and a combination of antimycin A/rotenone were injected through ports in the Seahorse Flux Pak cartridges to final concentrations of 4 μ g/mL, 4 μ mol/L, and 10 μ mol/L/1 μ mol/L, respectively.²⁹ Figure 1 illustrates how basal (baseline), ATP, non-ATP, and nonmitochondrial levels of oxygen consumption rates (OCRs) are evaluated. Baseline OCR ($OCR_{baseline}$) is represented by the total OCR of the cells before addition of mitochondrial inhibitors. Because oligomycin inhibits mitochondrial ATP synthase (complex V), non-ATP-linked OCR is equal to the rate observed in the presence of oligomycin (OCR_{oligo}). Consequently, ATP-linked OCR (OCR_{ATP}) is determined by subtracting the non-ATP OCR from the baseline OCR ($OCR_{ATP} = OCR_{baseline} - OCR_{oligo}$). Maximal OCR (OCR_{max}) is determined in the presence of carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone, an ionophore that uncouples the mitochondrion, leading to maximal oxygen consumption in an attempt to re-establish a membrane potential. Finally, addition of antimycin A and rotenone blocks electron entry into the electron transport chain, inhibiting mitochondrial oxygen consumption, which allows estimation of nonmitochondrial OCR, which is subtracted out as background for the mitochondrial OCR calculations. By using these OCR calculations, oxygen utilization can be estimated by determining the percentage of oxygen dedicated for ATP production using the formula $OCR_{ATP}/OCR_{baseline}$ and percent oxygen consumed for non-ATP purposes by using the formula $OCR_{oligo}/OCR_{baseline}$.

Determination of Intracellular ATP

For the determination of intracellular ATP, HUVECs were seeded at 30000 cells per well in 96-well tissue culture plates, and ATP levels were determined using the ATP bioluminescent somatic cell assay kit (Sigma, St. Louis, MO) following the manufacturer's instructions. ATP production rates were determined by measuring intracellular ATP under both basal and oligomycin (4 μ g/mL)-treated conditions.

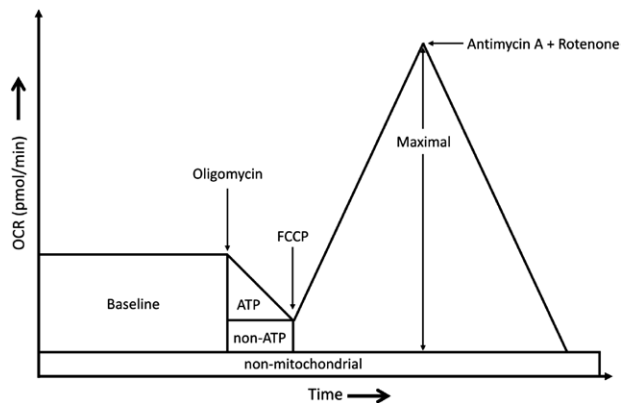


Figure 1. Experimental profile of basal, oligomycin, carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP), and antimycin A+rotenone-induced oxygen consumption rates (OCRs). For these studies, 30 000 human umbilical vein endothelial cells per well from single donors were cultured for 24 hours onto V7 tissue culture plates and OCRs were determined using the Seahorse XF24 Bioanalyzer. First, baseline OCRs (portion indicated by baseline) were determined. Second, oligomycin was added to inhibit mitochondrial respiration at ATP synthase and thus, decrease OCR by the amount of oxygen consumed for mitochondrial ATP production (indicated by ATP). The OCR remaining in the presence of oligomycin represents the oxygen consumed by the mitochondrion for non-ATP-related consumption (indicated by non-ATP) and nonmitochondrial oxygen consumption. Third, FCCP, an ionophore, uncouples mitochondrial respiration and dissipates mitochondrial membrane potential that induces maximal mitochondrial oxygen consumption (indicated by maximal). Finally, cells are treated with the combination of antimycin A and rotenone, inhibitors of cytochromes b and c and nicotinamide adenine dinucleotide dehydrogenase, respectively, which inhibits electron entry into electron transport and thus provides an estimate of nonmitochondrial oxygen consumption (indicated by nonmitochondrial).

Flow Cytometry

Cultured HUVECs grown to $\approx 85\%$ to 90% confluence in T-75 flasks were washed twice with PBS followed by a 5-minute incubation with 3 mL of 0.25% trypsin/EDTA at 37°C . After trypsin treatment, cells were resuspended in PBS at a final concentration of 300 000 cells per mL. MitoTracker Red was added to a final concentration of 200 nmol/L, and the mitochondria were labeled for 30 minutes at 37°C . After labeling, cells were washed once with PBS containing 5% FBS and then resuspended in 200 mL of PBS with 5% FBS for fluorescence-activated cell sorting analysis. Cells were analyzed with the assistance of the Analytic and Preparative Flow Core Facility on a SORP-LSRII digital cell analyzer, and MitoTracker Red fluorescence was monitored using the 535-nm excitation laser. Live cells were gated using FSC v. SSC and comparisons on the amount of MitoTracker Red staining compared with a nonstained negative control.

DNA Isolation and MtDNA Damage Assays

DNA was isolated from HUVECs or PBMCs. MtDNA copy number, quantitative polymerase chain reaction conditions, and calculation of DNA lesion frequencies have been previously described.^{30,31} Briefly, genomic DNAs were extracted using QIAamp DNA Mini kits (Qiagen), and mtDNA damage was assessed via amplification of a 16.2-kb quantitative polymerase chain reaction product (L) from mtDNA templates that was normalized by mtDNA copy number as determined by a smaller quantitative polymerase chain reaction product (S), yielding an L/S ratio.^{30,31} For the studies herein, mtDNA lesion frequencies were determined relative to either average L/S of haplogroup H or *DdeI*- (10398A) haplogroups, which represented zero-class lesions as previously described.³¹

Aconitase Assay

Aconitase activity was determined by measuring the transformation of isocitrate to *cis*-aconitate at 240 nm in 50 mmol/L Tris-HCl (pH 7.4) containing MnCl_2 and 20 mmol/L isocitrate at 25°C . Aconitase is specifically inactivated by superoxide ($\text{O}_2^{\cdot-}$) and peroxynitrite (ONOO^-), and hence, decreased activity correlates with increased oxidative stress associated with these oxidants.³²⁻³⁴ Because ONOO^- formation is related to $\text{O}_2^{\cdot-}$ production, decreased aconitase activity can be associated indirectly with increased $\text{O}_2^{\cdot-}$ generation. Decreases in enzymatic activity were interpreted as consistent with increased oxidant stress associated with $\text{O}_2^{\cdot-}$.

Human Subjects

To investigate the relationships between mtDNA haplogroups in terms of mtDNA damage in normal, healthy individuals, PBMCs from healthy, age-matched individuals (23 men and 22 women) were collected, and the mtDNA haplogroup was determined. MtDNA haplogroups were then segregated by the presence (+) or absence (-) of a *DdeI* restriction site at nucleotide pair 10394. An ancient transitional mutation, the guanosine (*DdeI*⁺) to adenosine (*DdeI*⁻) switch at nucleotide pair 10398 alters the last nucleotide of the *DdeI* recognition sequence (CTGAG) that begins at nucleotide pair 10394. This site is present in maternal lineages that are ubiquitous in African populations (L0 to L7) and also in a specific subset of West Eurasian mtDNAs (I, J, and K) proposed to have Middle Eastern origins but is absent in the remaining West Eurasian maternal lineages (haplogroups H, T, U, V, W, and X).^{35,36} Although this SNP has also been proposed to increase the risk of invasive breast cancer in black women when cancer exists, many studies investigating this issue have been conflicting.³⁷⁻⁴¹ Its use in the study herein was simply to segregate haplogroups into northern (*DdeI*⁻) or southern (*DdeI*⁺) latitudes. PBMCs were isolated from whole blood by density gradient centrifugation (BD Vacutainer CPT tubes Becton; Dickerson and Company, Franklin, NJ). The mean ages for individuals representing the *DdeI*⁻ ($n=29$) and *DdeI*⁺ ($n=16$) groups were 52.2 ± 1.4 and 50.4 ± 2.0 years, respectively ($P=0.47$).

To determine whether a relationship existed between mtDNA damage, CVD, and vascular function, an additional age-matched 56 subjects (23 healthy volunteers and 33 angiographically proven coronary artery disease) receiving care at Boston Medical Center were enrolled (mean age, 55.0 ± 10.0 years; 37 men and 19 women). Among these, 23 were healthy volunteers with no history of CVD, diabetes mellitus, hypertension, dyslipidemia, or cigarette smoking (10 men and 13 women), and 33 had angiographically proven coronary artery disease (27 men and 6 women). A blood sample was collected for the assessment of mtDNA damage, haplogroup status, flow-mediated dilation (FMD; a measure of endothelial vasodilator function), and brachial artery diameter (a measure of chronic remodeling) as previously described.⁴²⁻⁴⁴ All participants provided written informed consent. The protocol was approved by the Institutional Review Boards of the Boston Medical Center and the University of Alabama at Birmingham.

Vascular Measurements in Human Subjects

We examined the correlation between mtDNA damage and FMD (a measure of endothelial vasodilator function) and brachial artery diameter (a measure of chronic remodeling). Both variables have been shown to be related to cardiovascular risk.⁴⁵ Subjects rested for 10 minutes in a recumbent position, and endothelium-dependent brachial artery FMD was assessed as previously described.^{43,44} Briefly, 2-dimensional images and Doppler ultrasound signals were recorded from the brachial artery before and 1 minute after induction of reactive hyperemia by 5-minute cuff occlusion of the upper arm.

Statistical Analysis

Study results are expressed as mean \pm SEM. ANOVA was used to test the null hypothesis that all samples were drawn from a single population. If this test revealed significant differences ($P < 0.05$), then a Student-Newman-Keuls test was used for group comparisons. The

nonparametric Mann–Whitney rank sum test was used in certain cases (aconitase activity and mtDNA damage in HUVECs). We calculated Pearson correlations between the measures of vascular function and mtDNA damage. Statistical analyses were performed with SigmaStat statistical software (Systat Software, Inc).

Results

Single-Donor HUVECs From Haplogroup H or L Have Distinct Oxygen Consumption Profiles

OCRs were monitored in single-donor HUVECs representing haplogroups H and L under basal conditions and after treatment with oligomycin, carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone, and a combination of antimycin A+rotenone (design illustrated in Figure 1). Figure 2A shows that HUVECs from haplogroup H had significantly higher basal OCRs compared with those from haplogroup L (62.78±5.05 versus 48.60±2.40 pmol/min, respectively). Treatment with oligomycin, an inhibitor of complex V (ATP synthase), decreased OCRs in both haplogroups (Figure 2A; 24.17±4.19 versus 23.28±1.80 pmol/min); decreases in intracellular ATP levels were also observed for haplogroups H and L under these conditions (Figure 2B; 26.33%±7.15% versus 26.93%±6.07% in H versus L, respectively), suggesting similar patterns of ATP utilization for them. Dissipation of mitochondrial membrane potential by the addition of carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone revealed that HUVECs from haplogroup H had a significantly higher maximal OCR compared with those from haplogroup L (Figure 2A; 107.68±10.06 versus 69.89±5.49 pmol/min, respectively), whereas the addition of antimycin A+rotenone (which blocks mitochondrial electron flow) revealed no significant

differences in nonmitochondrial oxygen consumption between groups (Figure 2A; 24.36±5.43 versus 21.17±4.63 pmol/min, respectively). No differences in the mitochondrial number per cell were observed between haplogroups H and L (Figure 2C), suggesting that the differences in oxygen consumption were not because of the organelle number per cell between the 2 lineages.

Oxygen Utilization Differs in HUVECs From Haplogroups H and L

HUVECs from haplogroups H and L maintained similar steady-state levels of intracellular ATP (Figure 3A) under basal conditions although those from haplogroup L trended toward increased levels of intracellular ATP (344.12±53.56 pmol/3×10⁴ cells versus 442.98±31.71 pmol/3×10⁴ cells in H and L, respectively). Because HUVECs from the 2 haplogroups had significantly different OCRs yet maintained similar levels of intracellular ATP, we postulated that they had different oxygen utilization profiles. Consequently, the percentage of oxygen consumed for ATP production relative to total oxygen consumption was calculated in HUVECs from haplogroups H and L. Using oligomycin as a specific inhibitor of ATP synthase, the percentage of oxygen dedicated for ATP production is calculated by subtracting the oligomycin-inhibited OCR from the baseline OCR ($OCR_{ATP} = OCR_{baseline} - OCR_{oligo}$) and dividing by the baseline rate ($OCR_{ATP} / OCR_{baseline}$; Methods section). This estimates oxygen consumption directly linked to mitochondrial ATP synthesis relative to all other oxygen consuming processes in the mitochondria (eg, proton leak and oxidant generation). Figure 3B shows that HUVECs from the 2 haplogroups had significant differences in the percentage of

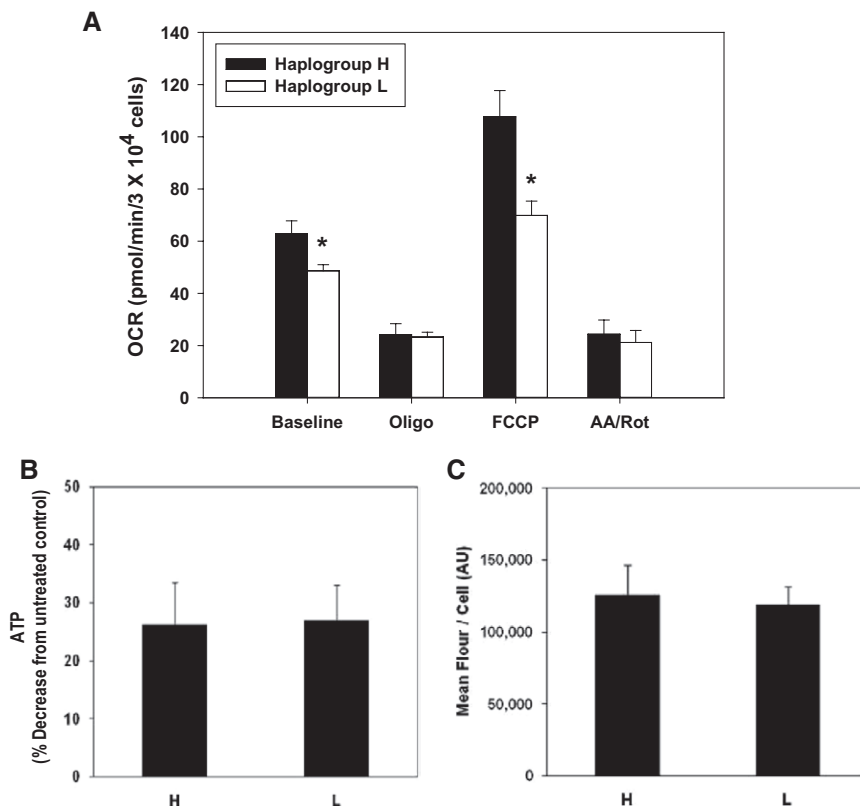


Figure 2. Oxygen consumption rates (OCRs), ATP utilization, and the mitochondrial number in human umbilical vein endothelial cells (HUVECs) from haplogroups H and L. **A**, HUVECs from haplogroup H (West Eurasian; n=5 single donors) and haplogroup L (African; n=9 single donors) were seeded onto Seahorse XF24 plates (30 000 cells per well), and OCRs were monitored at baseline and after treatment with oligomycin (4 µg/mL), carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP; 4 µmol/L), or antimycin A (AA)+rotenone (Rot; 10 and 1 µmol/L) for a period of 18 minutes in unbuffered minimal essential media. **B**, Under identical conditions, the decrease in intracellular ATP because of oligomycin treatment (4 µg/mL) was determined relative to untreated controls. **C**, Mitochondrial number was assessed by staining mitochondria from both groups with MitoTracker Red (200 nmol/L) for 30 minutes and determining the average fluorescence per cell via flow cytometry. Values are mean±SEM of ≥3 independent experiments per single-donor cell line. *P<0.05.

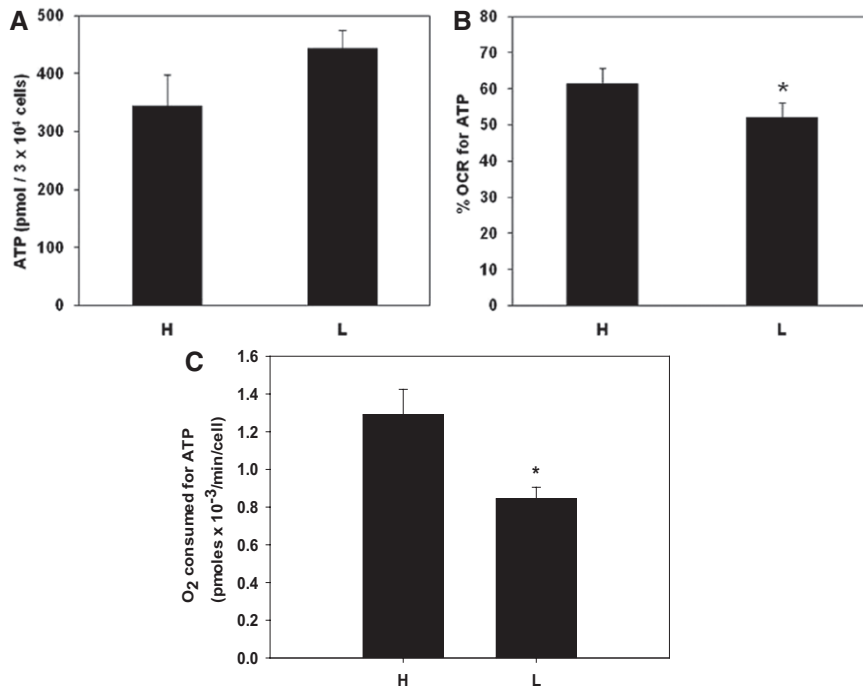


Figure 3. Oxygen consumption devoted to ATP maintenance differs in human umbilical vein endothelial cells (HUVECs) from haplogroups H and L. **A**, HUVECs from haplogroup H (West Eurasian) and haplogroup L (African) were seeded onto 96-well plates (30 000 cells per well), and steady-state concentrations of ATP were determined via bioluminescent detection (Methods section). **B**, Percent oxygen utilization for ATP production in HUVECs was calculated by subtracting oligomycin-associated oxygen consumption from baseline consumption and dividing by baseline consumption [(baseline–oligomycin)/baseline]. **C**, Cellular oxygen consumption rate (OCR) for ATP production was determined by multiplying the basal oxygen consumption rates by the proportion of oxygen used for ATP generation for each sample. Bar graph depicted the mean and SEM for all samples. Values presented as mean±SEM of ≥3 independent experiments per single-donor cell line (n=5 haplogroup H single donors and n=9 haplogroup L single donors). **P*<0.05.

basal oxygen used for ATP production: haplogroup H committed 61.5%±4.2% of total oxygen consumed under basal conditions for ATP production, whereas HUVECs in haplogroup L used 52.1%±4.1% of total oxygen consumed for ATP production. These results translated into haplogroup H HUVECs using more oxygen for ATP production than haplogroup L HUVECs under basal conditions (1.29×10⁻³ pmoles of oxygen per minute per cell versus 8.44×10⁻⁴ pmoles of oxygen per minute per cell, respectively; Figure 3C). Thus, haplogroup H HUVECs consumed more oxygen to maintain similar steady-state levels of ATP indicating a lower mitochondrial economy with respect to ATP production when compared with those from haplogroup L.

Mitochondrial Damage Is Increased in HUVECs From Haplogroup L

As these studies suggested that HUVECs from haplogroup L were more economical in generating ATP than those from haplogroup H, we were curious to know whether levels of mitochondrial damage were different between the 2 lineages. Consequently, activity of aconitase, a citric acid cycle enzyme in the mitochondrial matrix, which is specifically inactivated by superoxide or peroxynitrite,³³ was quantified to determine overall levels of oxidative stress within the mitochondrion. Figure 4A shows that aconitase activity was significantly decreased in HUVECs from haplogroup L compared with those in haplogroup H, indicating greater oxidative stress in the mitochondria from the former group. Next, aconitase protein levels were determined via immunoblot, and no significant differences between H and L haplogroups were observed (data not shown), indicating that the differences in enzyme activity were not because of differences in protein levels. To more specifically examine the degree of mtDNA damage in both groups, the level of mtDNA damage was determined in HUVECs from both haplogroups H and L. Consistent with the

aconitase results, Figure 4B shows that HUVECs from haplogroup L had significantly more mtDNA damage relative to those from haplogroup H (0.45±0.096 lesions per 16 kb versus 0.0±0.088 lesions per 16 kb, respectively). Collectively, these results suggest that HUVECs belonging to haplogroup L are exposed to higher levels of oxidant stress compared with those in haplogroup H, as reflected by differences in enzymatic activity of aconitase, and the levels of mtDNA damage.

MtDNA Damage Differs in PBMCs Collected From Individuals With Haplogroups Having African or West Eurasian Origins

To further investigate whether differences in mtDNA damage levels existed between humans having West Eurasian or African maternal origins, PBMC mtDNAs from 45 age-matched healthy individuals were sequenced and then placed into either African or West Eurasian maternal lineages. Interestingly, no differences in mtDNA damage between these 2 groups were initially observed (data not shown). However, additional analysis revealed that differences in mtDNA damage existed when haplogroups were segregated using the 10398 SNP resulting in the presence (+) or absence (–) of the *DdeI* 10394 site (Figure 5A). When the ancestral nucleotide (G) is present at 10398, it creates the *DdeI*⁺ 10394 site. African lineages (*DdeI*⁺) are distinguished from the bulk of West Eurasian (*DdeI*[–]) haplogroups (H, T, U, V, W, and X) by this site (the latter having 10398A). However, the site has also independently recurred in 3 West Eurasian haplogroups (I, J, and K) that are phylogenetically distinctive from each other, arose at different times in Europe or the Middle East, and have a more southern latitude distribution.⁴⁶ Further analysis showed that differences in mtDNA damage were more significantly associated with sex. *DdeI*⁺ 10394 men had significantly higher levels of mtDNA damage relative to *DdeI*[–] 10394 men (Figure 5B; 0.0±0.094 lesions per 16 kb versus 0.766±0.229 lesions per

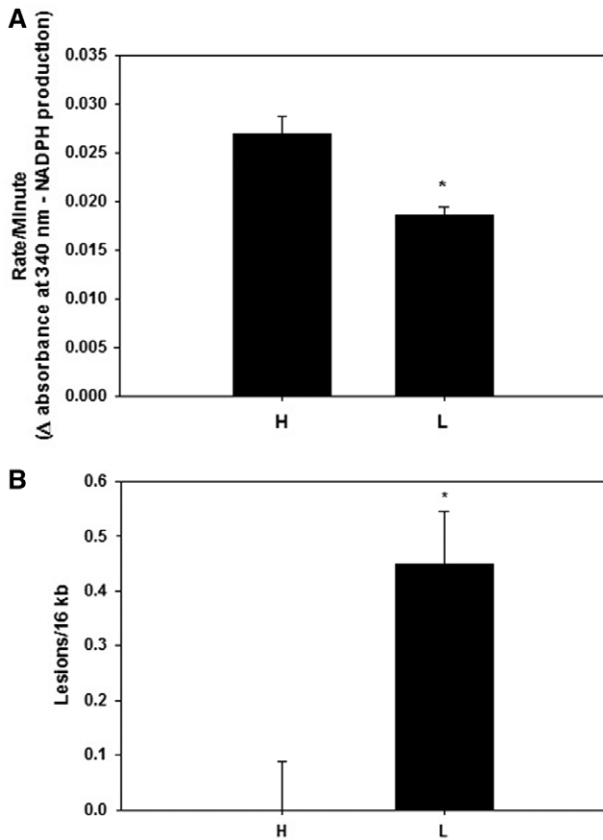


Figure 4. Mitochondrial DNA (mtDNA) damage in human umbilical vein endothelial cells (HUVECs). **A**, The activity of the citric acid cycle enzyme aconitase was quantified in HUVECs belonging to haplogroup H (West Eurasian; $n=3$ single donors) and haplogroup L (African; $n=3$ single donors) to determine relative levels of oxidative stress—aconitase is specifically inactivated by superoxide or peroxynitrite in the mitochondrial matrix. Decreased activity indicates increased oxidant stress. Data are presented as mean \pm SEM from 3 independent determinations per cell line. * $P<0.05$. **B**, Baseline mtDNA damage was assessed in HUVECs from haplogroup H (West Eurasian; $n=3$ single donors) and haplogroup L (African; $n=3$ single donors) via quantitative polymerase chain reaction. MtDNA damage is expressed relative to haplogroup H (zero-class lesions). Data are presented as mean \pm SEM and representative of ≥ 3 independent determinations per cell line. * $P<0.05$.

16 kb), whereas no significant differences were observed in healthy women (data not shown).

Vascular Function, Remodeling, and mtDNA Damage in Humans

Because increased ROS production contributes to both endothelial dysfunction and mtDNA damage, we were interested in determining whether a relationship existed between mtDNA damage, vascular function, and CVD. Consequently, mtDNA damage was assayed in PBMCs collected from 33 patients with CVD and 23 age-matched controls in which vascular function had also been assessed by FMD. Changes in brachial artery diameter were monitored in response to reactive hyperemia, whereas vascular remodeling was assessed by quantifying the brachial diameter in the conduit arteries of the upper extremity through pulse wave velocity. Figure 6A shows that a significantly positive relationship between brachial diameter

and mtDNA damage was observed in all subjects ($r=0.42$; $P=0.001$). Consistent with pathological changes in vascular function, a negative relationship between FMD and mtDNA damage was also observed in all subjects (Figure 6B; $r=-0.32$; $P=0.01$).

To further determine whether PBMC mtDNA damage was also associated with CVD, levels of mtDNA damage were compared between patients and age-matched, healthy controls. The results showed that patients with CVD had significantly higher levels of mtDNA damage in their PBMCs compared with age-matched controls (Figure 6C; 0.686 ± 0.0882 versus 0.00 ± 0.115 , respectively). These differences also seemed to be maintained between the *DdeI* 10394 (–) and (+) haplogroups compared with their corresponding age-matched controls (Figure 6D). As found previously, an interaction between latitude and sex was also observed in that mtDNA damage was significantly higher in the healthy male control *DdeI*⁺ 10394

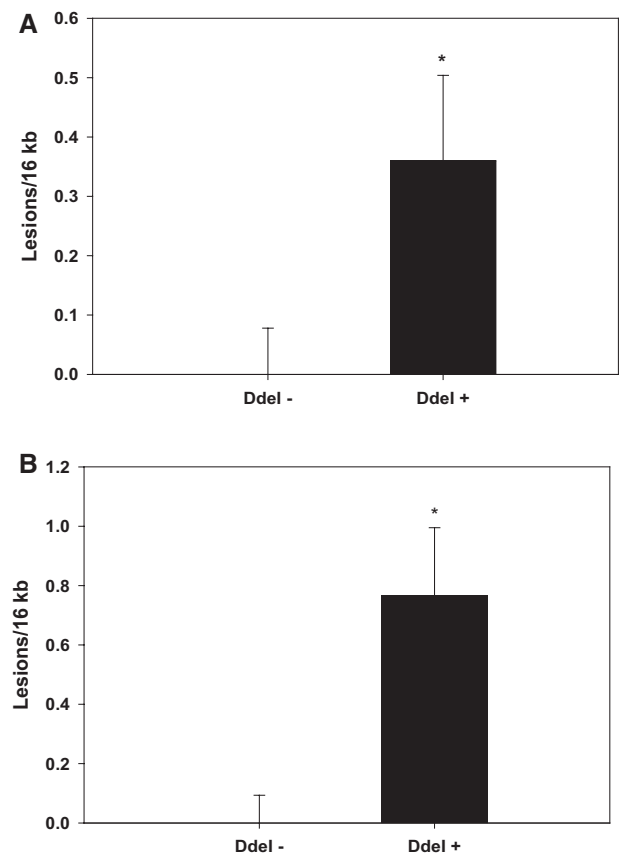


Figure 5. Mitochondrial DNA (mtDNA) damage in healthy humans. **A**, MtDNA damage was quantified from genomic DNA extracted from peripheral blood mononuclear cells (PBMCs) collected from 45 age-matched, healthy controls that were positive (*DdeI*⁺) or negative (*DdeI*⁻) for a *DdeI* 10394 restriction site that delineates African ($n=6$) or West Eurasian ($n=29$) mtDNA haplogroups. MtDNA damage is expressed relative to the *DdeI*⁻ haplogroups (zero-class lesions). Data are presented as mean \pm SEM. **B**, MtDNA damage was quantified from genomic DNA extracted from PBMCs collected from 23 age-matched, healthy male controls who were positive (*DdeI*⁺) or negative (*DdeI*⁻) for the *DdeI* 10394 restriction site, indicative of mtDNA haplogroups of African ($n=8$) or West Eurasian ($n=15$) origin. MtDNA damage is expressed relative to the *DdeI*⁻ haplogroups (zero-class lesions). Data are presented as mean \pm SEM. * $P<0.05$.

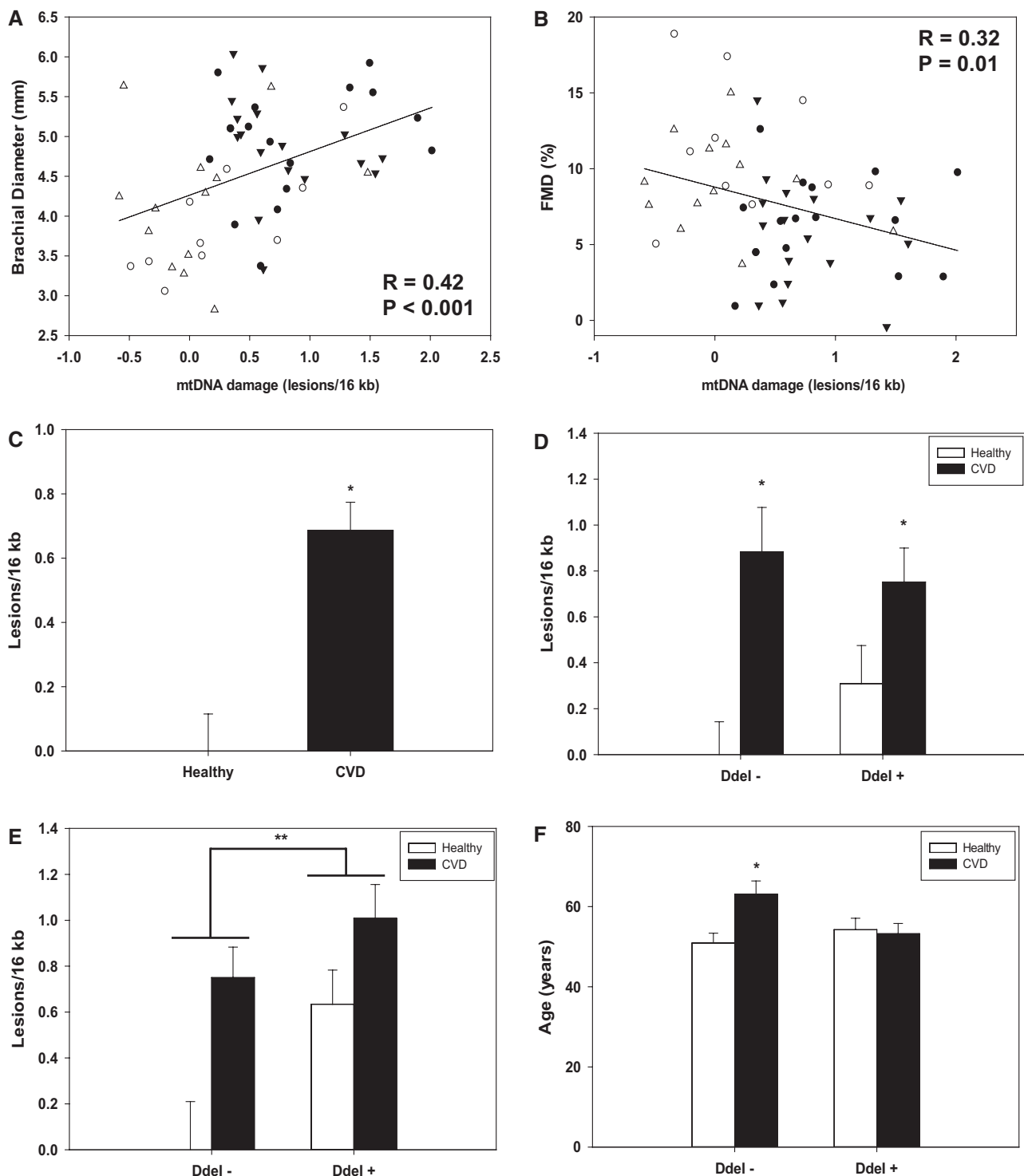


Figure 6. Relationship between mitochondrial DNA (mtDNA) damage and markers of vascular dysfunction. Brachial diameter and flow-mediated dilation (FMD) were determined in 34 patients with cardiovascular disease (CVD; filled symbols) and 23 age-matched control (open symbols) human subjects, and the relationship with peripheral blood mononuclear cell (PBMC) mtDNA damage was examined. Circles and triangles indicate Ddel⁺ (open circles: healthy controls [n=10]; filled circles: CVD [n=17]) and Ddel⁻ (open triangles: healthy [n=13]; filled triangles: CVD [n=17]) mtDNA haplogroups, respectively. **A**, A significant positive relationship was found between brachial diameter and mtDNA damage. **B**, A significant negative relationship was identified between FMD and mtDNA damage. **C**, Mean PBMC mtDNA damage levels were significantly different between patients with CVD and age-matched controls. MtDNA damage is expressed relative to healthy controls (zero-class lesions). * $P < 0.001$. **D**, MtDNA damage was quantified from genomic DNA extracted from PBMCs collected from CVD age-matched, healthy controls that were positive (Ddel⁺) or negative (Ddel⁻) for a Ddel 10394 restriction site. MtDNA damage is expressed relative to the Ddel⁻ healthy controls (zero-class lesions). Data are presented as mean \pm SEM. * $P < 0.05$ from matched healthy controls. **E**, MtDNA damage in Ddel⁻ 10394 (healthy controls, n=6; patients with CVD, n=15) and Ddel⁺ 10394 (healthy controls, n=4; patients with CVD, n=13) male patients with CVD and healthy controls. Data are expressed relative to the Ddel⁻ healthy control group (zero-class lesions). * $P < 0.05$ between patients with CVD and matched healthy controls, ** $P < 0.05$ between Ddel 10394 (-) versus (+) groups (healthy+CVD combined). **F**, Mean age of controls and patients with CVD in Ddel⁻ (healthy controls, n=13; patients with CVD, n=17) and Ddel⁺ (healthy controls, n=10; patients with CVD, n=17) individuals. * $P < 0.05$ from healthy control.

lineages compared with those with *DdeI*⁻ 10394 haplogroups ($P=0.018$). In addition, these differences were also seen when comparing overall haplogroups irrespective of health status (eg, healthy+CVD *DdeI*⁻ 10394 versus healthy+CVD *DdeI*⁺ 10394; $P=0.021$; Figure 6E). Although differences between healthy and CVD groups were maintained within *DdeI*⁻ 10394 and *DdeI*⁺ 10394 haplogroups in women, no significant association was observed for women on latitude within the healthy or CVD groups (data not shown).

Finally, although the mean ages of healthy and CVD groups were not significantly different when undifferentiated by the mtDNA haplogroup, further analysis revealed an interesting relationship between the ages of healthy controls, patients with CVD, and *DdeI* 10394 status. Figure 6F shows that the age of patients with CVD was significantly older in individuals who belonged to *DdeI*⁻ 10394 haplogroups relative to (*DdeI*⁻) healthy controls (12.15 years older) and their *DdeI*⁺ CVD counterparts (9.82 years older), consistent with the notion of a delayed onset of CVD for *DdeI*⁻ 10394 haplogroup individuals.

Discussion

Numerous epidemiological studies have demonstrated that associations exist between the mitochondrial haplogroup and the incidence of disease,^{7–9,47–49} and more recent studies in animals have shown that mtDNA background can play a significant role in aspects of disease susceptibility in the heart and liver.^{18,19} Whether differences exist in cellular bioenergetics and mitochondrial damage between normal individuals having different mtDNA backgrounds has not been studied in primary cells. In this study, primary endothelial cells having mtDNAs belonging to either H (West Eurasian) or L (African) haplogroups exhibited significant differences in mitochondrial function, oxygen utilization, and damage consistent with the observed differences in CVD susceptibility between individuals with West Eurasian (eg, European) and African ancestries.

Classically, the identification of mtDNA haplogroups was based on the presence or absence of different diagnostic SNPs that had been identified in numerous molecular anthropology and evolutionary studies.^{36,50,51} The reckoning of these SNPs and the haplogroup classifications they facilitated were made through comparisons with the Cambridge Reference Sequence for the human mtDNA,⁵² which was later revised to correct for sequencing errors and haplogroup-specific markers.⁵³ A more refined haplogroup classification was achieved through the comparison of whole-mtDNA genome sequences from various world populations^{26,54–57} and the comparison of control region (D-loop) sequences from previous mtDNA studies to these new whole-mtDNA genome data sets.⁵⁸ In this context, it was demonstrated that populations harboring mtDNA haplogroups from more northern latitudes had a greater proportion of missense mutations relative to those living in Africa.³⁵ On the basis of this observation, it was hypothesized that the increase in missense mutations in certain East and West Eurasian haplogroups allowed for better adaptation and survival of human populations in colder climates because of increased heat production resulting from less-efficient energy coupling. By contrast, haplogroups arising in populations living in sub-Saharan Africa were predicted to be more economical in the use of

caloric energy for generation of molecular energy (ATP).^{35,59,60} The data presented in this study are consistent with the latter view in that cells having L mtDNAs generate equal amounts of ATP compared with those harboring H mtDNA yet use less oxygen. These findings are also consistent with the prediction that individuals with more economical mitochondria will be more prone to oxidative stress compared with those with less economical mitochondria (eg, haplogroup H). They are further consistent with mitochondrial paradigms that propose that different mtDNA lineages convey distinct mitochondrial bioenergetic capacities because of prehistoric selection events for features of mitochondrial function.⁶¹

Interestingly, women of African descent have generally lower values of total and resting daily energy expenditure rates compared with white women,^{62–64} and in this respect, uncoupling protein (UCP) gene variation has been interrogated as a potential basis for these differences.⁶⁵ However, no significant variation in resting daily energy expenditure has been linked with UCP gene variants with exception of an exon 5 variant for UCP3, and although this variant is found in both women of African and Eurasian descent, the association is only found in those of African ancestry.⁶⁵ Although UCP expression and genetic variability were not investigated herein, no differences in OCR were observed between or within the H and L haplogroups in the presence of oligomycin or antimycin A+rotenone (Figure 2A), suggesting little or no role of UCPs in the differences seen in HUVEC experiments herein—differential UCP expression should manifest in differences in OCRs in the presence of oligomycin—furthermore, the lack of differences in OCR between oligomycin and antimycin A+rotenone suggests that the observed differences in HUVECs are not consistent with differential UCP expression. Finally, the 10398 SNP used to segregate mtDNA haplogroups into northern and southern latitudes has been previously associated with certain neurodegenerative diseases and breast cancer.^{38,66} However, these findings are controversial,^{37,40,41} and whether this SNP is directly related to the differences observed in these studies is not yet known. Direct comparisons between closely related mtDNA haplogroups within the West Eurasia may provide additional insights into this question.

Subtle differences in mitochondrial function in endothelial cells could potentially contribute to CVD initiation and progression through the production of ROS. Measures of mtDNA damage in both cultured cells (in vitro) and tissues (in vivo) are consistent with this notion.^{19,42,67–69} Furthermore, these differences in function are likely to be exacerbated under conditions of positive energy balance (excess substrate [high caloric intake] and low energy demand [physical inactivity]) and cellular challenges (eg, inflammation), which increase the propensity for mitochondrial ROS production. The observations of increased oxidative stress (aconitase activity) and mtDNA damage in HUVECs harboring haplogroup L mtDNAs relative to those having haplogroup H mtDNAs support this concept. However, under conditions of chronic stress, even individuals having haplogroup H mtDNAs would sustain significant damage over time and therefore be also predisposed to eventual CVD development. Our observations that the average age of patients with CVD is significantly older in *DdeI*⁻ individuals are consistent with this notion, as are

the findings of greater levels of mtDNA damage in healthy individuals harboring *DdeI*⁺ haplogroups compared with those with *DdeI*⁻ lineages. Consequently, it follows that differences in mitochondrial function and damage may only be observed in healthy controls and will not be easily observable in individuals with advanced CVD belonging to different mtDNA haplogroups. Interestingly, comparison of vascular function (FMD) with the level of mtDNA damage present in lymphocytes collected from patients with CVD and healthy controls revealed a significant relationship between mtDNA damage and decreased vessel relaxation (Figure 6B), accompanied by significant changes in vascular remodeling (Figure 6A). These findings suggest that perhaps mtDNA integrity of the circulating blood cells can serve as an indirect and early biomarker of vascular function and condition. Accordingly, information on an individual's haplotype (haplogroup), bioenergetic profile, and mtDNA integrity (mtDNA damage) will be required for a more complete assessment of vascular disease risk. Future studies that pursue understanding the interrelationship between genetics, bioenergetics, and cellular integrity will provide a clear basis for making informed decisions on individual risk for CVD development.

Studies illustrating the mitochondrion's role in many disease processes continue to grow. The data presented here demonstrate that endothelial cell mtDNAs from haplogroups H and L, which are maternal lineages with distinct geographic origins (West Eurasia and Africa), have unique mitochondrial bioenergetic profiles. We show that these differences also seem to associate with levels of mtDNA damage and oxidant stress. Additional analyses in blood samples from healthy humans showed that mtDNA damage was higher in *DdeI*⁺ haplogroups compared with *DdeI*⁻ haplogroups and also higher in patients with CVD compared with healthy controls. Consequently, when making comparisons between healthy and diseased groups, it seems advisable to perform haplogroup matching between age-matched controls and disease cohorts whenever possible. Increases in mtDNA damage were also associated with decreased FMD and increased vascular remodeling in humans, and was also observed to be highest in CVD patients. This work demonstrates that subtle differences in bioenergetics between mitochondrial haplogroups occur in primary cells. These effects may have a significant influence on endothelial cell function which, in turn, can affect systemic vascular function. The molecular mechanisms behind these effects are currently unclear; it is unlikely, however, that a singular molecular pathway will be the basis because of the nature of mitochondrial metabolism which is at the hub of all cellular metabolism, and therefore, even subtle changes have the potential for affecting multiple aspects of cellular function. Studies using animal models that examine the effect of different normal mtDNA backgrounds have shown effects on adaptive immunity, cognition, heart failure susceptibility, fatty liver disease, and most recently cancer.^{13,18,19,70,71} The diversity of these phenotypes suggests that a single mechanism is unlikely and instead argues that a complex interaction of mitochondrial and nuclear genetic backgrounds guides cellular metabolism in response to environmental cues and challenges which influences cellular function in response.

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Disclosures

None.

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CLINICAL PERSPECTIVE

Studies illustrating the mitochondrion's role in many disease processes continue to grow—the data herein demonstrate that endothelial cells derived from maternal lineages with distinct geographic origins (West Eurasia and Africa) have unique bioenergetic profiles that also associate with differential levels of mitochondrial DNA (mtDNA) damage and oxidant stress. Analyses using blood samples from healthy humans showed that mtDNA damage was higher in individuals having mtDNAs representative of southern geographic origins (Africa) compared with those having northern origins (West Eurasia) and that patients with cardiovascular disease had increased mtDNA damage compared with matched, healthy controls. Increases in mtDNA damage were also associated with decreased flow-mediated dilation and increased vascular remodeling in humans, and was also observed to be highest in cardiovascular disease patients. This work demonstrates that subtle differences in bioenergetics between mitochondrial haplogroups occur in primary cells that may have a significant influence on vascular function. The molecular cause behind these effects is currently unclear; however, it is unlikely that a single mechanism is causative, and instead, we propose that a complex interaction of mitochondrial and nuclear genetic backgrounds guides cellular metabolism in response to environmental cues and challenges that influence cellular function in response. This concept of Mito-Mendelian interactions could explain the genetics of common disease susceptibility and therefore may be a potentially important clinical consideration when assessing risk.

Endothelial Cell Bioenergetics and Mitochondrial DNA Damage Differ in Humans Having African or West Eurasian Maternal Ancestry

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