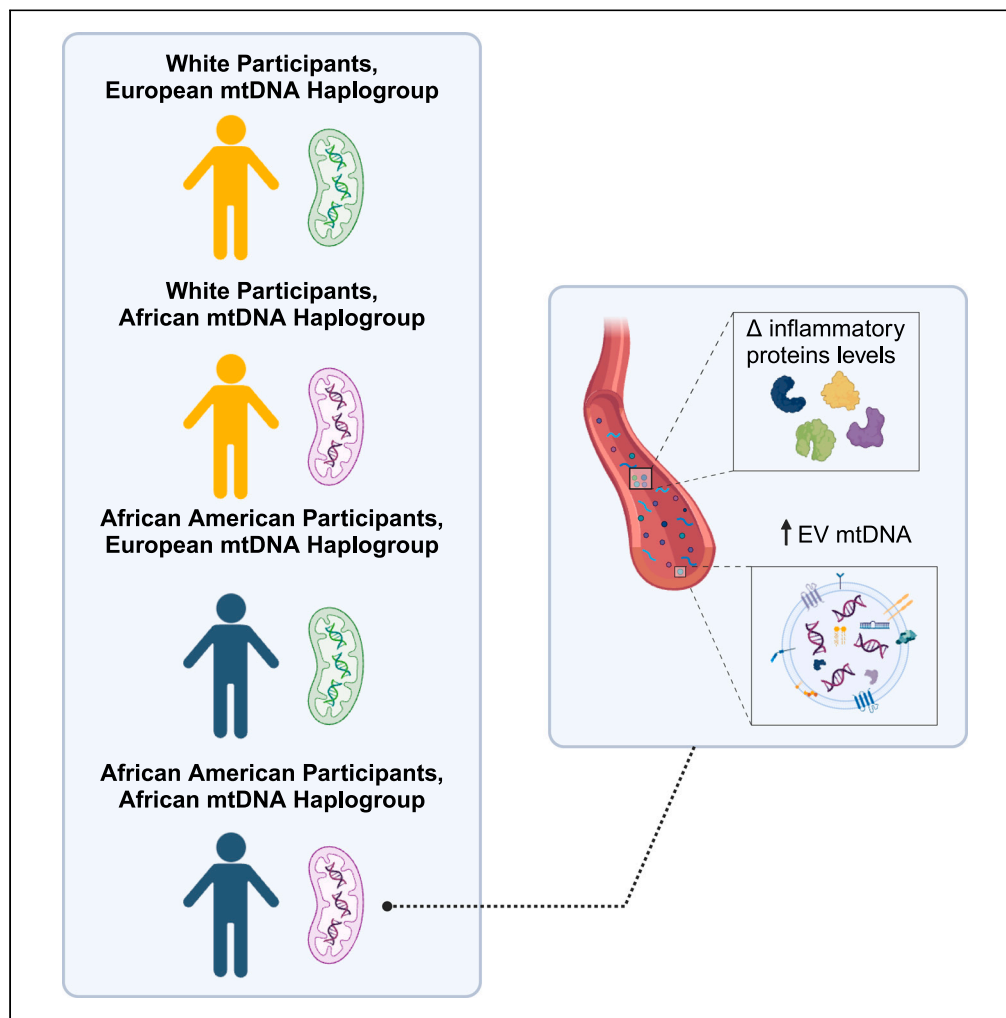## Article

# Extracellular vesicle mitochondrial DNA levels are associated with race and mitochondrial DNA haplogroup

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### Highlights

ccf-mtDNA in plasma and
EVs were higher in African
American adults

ccf-mtDNA in plasma and
EVs were not associated
with hypertension

EV mtDNA was highest in
African American adults
with African mtDNA
haplogroup

Inflammatory proteins
differed by mtDNA
haplogroup, race, and EV
mtDNA

## Article

# Extracellular vesicle mitochondrial DNA levels are associated with race and mitochondrial DNA haplogroup

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## SUMMARY

**Circulating cell-free mitochondrial DNA (ccf-mtDNA) acts as a damage-associated molecular pattern molecule and may be cargo within extracellular vesicles (EVs). ccf-mtDNA and select mitochondrial DNA (mtDNA) haplogroups are associated with cardiovascular disease. We hypothesized that ccf-mtDNA and plasma EV mtDNA would be associated with hypertension, sex, self-identified race, and mtDNA haplogroup ancestry. Participants were normotensive (n = 107) and hypertensive (n = 108) African American and White adults from the Healthy Aging in Neighborhoods of Diversity across the Life Span study. ccf-mtDNA levels were higher in African American participants compared with White participants in both plasma and EVs, but ccf-mtDNA levels were not related to hypertension. EV mtDNA levels were highest in African American participants with African mtDNA haplogroup. Circulating inflammatory protein levels were altered with mtDNA haplogroup, race, and EV mtDNA. Our findings highlight that race is a social construct and that ancestry is crucial when examining health and biomarker differences between groups.**

## INTRODUCTION

Damage-associated molecular pattern (DAMP) molecules are released because of cellular stress and damage, trauma, ischemia, or other environmental circumstances and can drive sterile, pathogen-free inflammation.[1,2] These endogenous signals can include proteins like high-mobility group box 1 (HMGB1), reactive oxygen species (ROS) released from damaged mitochondria, as well as circulating cell-free mitochondrial DNA (ccf-mtDNA).[2–4] These DAMPs are recognized by certain receptors such as Toll-like receptors (TLRs), which initiate pro-inflammatory signal transduction pathways.[1,5] Chronic inflammation associated with DAMPS can lead to the development of age-related and inflammatory-associated diseases, such as cardiovascular disease (CVD) and diabetes.[1,5–7]

Although a major preventable driver of CVD, hypertension remains the leading risk factor for global morbidity and mortality.[8–10] Additionally, the incidence of hypertension is highest in Black men.[11] Few studies have explored the role of ccf-mtDNA in the context of hypertension.[7,12–14] mtDNA levels of the mitochondrially encoded NADH dehydrogenase 1 and 6 (*MT-ND1*, *MT-ND6*) are significantly higher in the plasma of patients with resistant hypertension (n = 91) compared with controls (n = 37), with mtDNA levels significantly higher in Black patients (n = 40) compared with White patients (n = 51) with resistant hypertension.[13] McCarthy et al. have shown that levels of ccf-mtDNA in rats, specifically the cytochrome *b*, mitochondrial (*mt-Cytb*), and *mt-Nd6* gene regions, are elevated in male rats with spontaneous hypertension (n = 3–8).[14] Based on these findings, McCarthy et al. hypothesized that acute hypertension in football players may be driven by musculoskeletal injury from repeated hits, leading to elevated levels of HMGB1 and ccf-mtDNA.[12,15] These DAMPs then go on to activate TLRs on endothelial cells, leading to endothelial dysfunction and hypertension.[12]

Ccf-mtDNA in plasma can be carried in extracellular vesicles (EVs).[16–19] EVs are small, membrane-bound vesicles that can carry various cargo including nucleic acids, lipids, and proteins.[20–22] They are involved in intercellular communication and therefore play a role in the pathophysiology of numerous diseases.[23] There are different types of EVs, including exosomes (~30–150 nm), microvesicles (~100–1000 nm), and apoptotic bodies (~1000–5000 nm), differing by their size and biogenesis pathways.[24] Due to the difficulty in deciphering the biogenesis pathway, the collective term of EV is used.[25] EVs can be readily isolated from bodily fluids such as plasma and urine and therefore are currently studied for their biomarker and therapeutic potential in several diseases, including CVD.[26,27] EVs have also been shown to play a functional role in CVD. Yadid et al. have shown that treatment of cardiac tissue with endothelial-cell-derived EVs (EEVs) alleviated cell death as well as loss of contractile capacity during and after ischemic reperfusion injury.[28] Additionally, EEVs from obese/hypertensive adults were shown to

**Table 1. Cohort demographics**

| Characteristic | NT, N = 107 | HTN, N = 108 | p value |
|---|---|---|---|
| Age | 52.6 (4.3) | 53.1 (4.1) | 0.3 |
| AA (%) | 54 (50%) | 54 (50%) | >0.9 |
| Men (%) | 54 (50%) | 54 (50%) | >0.9 |
| Below poverty (%) | 46 (43%) | 47 (44%) | >0.9 |
| Hypertension medication | 0 (0%) | 74 (69%) | <0.001 |
| Systolic BP (mmHg) | 109.6 (11.6) | 121.2 (17.9) | <0.001 |
| Diastolic BP (mmHg) | 63.9 (7.8) | 69.1 (10.1) | <0.001 |
| Haplogroup | | | 0.8 |
| African | 48 (45%) | 45 (42%) | |
| European | 54 (50%) | 56 (52%) | |
| Other | 5 (4.7%) | 7 (6.5%) | |

Age and blood pressure are reported as mean ± (SD), whereas N (%) are reported for race, sex, poverty status, hypertension medication use, and haplogroup. Student's t test was used to analyze differences between hypertension groups for age, systolic, and diastolic blood pressure. Pearson's chi-squared test was used to analyze differences for race, sex, poverty status, hypertension medication use, and haplogroup.

AA, African American; BP, blood pressure; mmHg, millimeters of mercury; NT, normotensive; HTN, hypertensive.

increase factors associated with hypertrophy (cardiac troponin T, α-actin, nuclear factor kB) and fibrosis (transforming growth factor (TGF) β, collagen1-α1), as well as endothelial nitric oxide synthase (eNOS) expression and nitric oxide (NO) production in cardiomyocytes.[29] However, little is known about the role of race and social determinants of health in EV pathophysiology in the context of CVD or specifically hypertension.

Previously, we have shown that EV-associated mtDNA (EV mtDNA) levels decline with age.[16] Additionally, we found that EV mtDNA levels for two gene regions, the mitochondrially encoded NADH dehydrogenase 2 (*MT-ND2*) and mitochondrially encoded cytochrome *c* oxidase subunit II (*MT-CO2*), were higher in frail individuals compared with non-frail individuals at mid-life.[18] The association of increased EV mtDNA with frailty is interesting, due to the risk of early mortality with frailty at mid-life.[30]

Mitochondrial dysfunction is linked to aging as well as diseases such as cardiovascular disorders, diabetes mellitus, Parkinson disease, and cancer.[31–34] Compared with nuclear DNA, mtDNA displays a higher mutation rate, potentially because mtDNA is closer in proximity to reactive oxygen species (ROS).[35,36] As mitochondria are maternally inherited without germline recombination, the accumulation of mutations such as single nucleotide polymorphisms (SNPs) in mtDNA can be tracked across human evolutionary history.[37,38] Sequentially accumulated SNPs thus define mitochondrial DNA haplogroups (mtDNA haplogroup) as a set of shared SNPs in a population.[39,40]

mtDNA haplogroups have been associated with differences in mitochondrial function. Studies performed on cytoplasmic hybrid (cybrid) cell lines, containing identical nuclear DNA but differing mtDNA, as well as primary cell lines have shown that mtDNA haplogroups are associated with differing levels of mtDNA damage, oxidative phosphorylation efficiency, and ROS production.[41–45] Mutations in mtDNA are linked to the manifestation of mitochondria-related diseases such as Leber hereditary optic neuropathy or mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes.[46,47] Cardiovascular involvement is prevalent in mitochondrial disease, with a previously measured 30% baseline prevalence of cardiac involvement in individuals with mitochondrial diseases.[47] mtDNA polymorphisms considered non-pathogenic have also been linked to an increased risk of CVD.[48–51] However, racial/ethnic diversity in cohorts studying the potential links between mtDNA haplogroups and CVD is limited.[49–52]

In our study, we hypothesized that (1) ccf-mtDNA in plasma or EVs may be associated with hypertension in our middle-aged cohort of African American and White participants, (2) mtDNA haplogroups may be associated with levels of ccf-mtDNA or EV mtDNA, and (3) EV mtDNA and/or mtDNA haplogroup may be influenced by sterile inflammation as measured by the presence of inflammatory markers.

## RESULTS

### Plasma EV characteristics

Recently, we reported that ccf-mtDNA can be encapsulated in EVs.[16,18] Therefore, we examined whether ccf-mtDNA in EVs may be altered with hypertension, race, and sex in this cohort. We studied middle-aged individuals (45–59 years) in the Healthy Aging in Neighborhoods of Diversity across the Life Span (HANDLS) study who were normotensive (n = 107) and hypertensive (n = 108) living above and below poverty, balanced across sex and self-identified race (African American or White; Table 1) hereafter referred to as race. Plasma EVs were isolated and analyzed according to the Minimal Information for Studies of Extracellular Vesicles (MISEV) guidelines from the International Society for Extracellular Vesicles.[53] Plasma EVs were first analyzed through immunoblotting against EV-associated markers including ALIX, Flotillin-1, CD9, and CD81 (Figure 1A). These markers were enriched in our EV samples and absent in the EV-depleted (EVD) samples. GM130 was used as a purity marker and was absent in the EV samples (Figure 1A). Transmission electron microscopy was then used to visualize the EVs and showed a typical EV morphology with intact vesicles (Figure 1B). Some vesicles were cup-shaped, which occurs during the dehydration process for
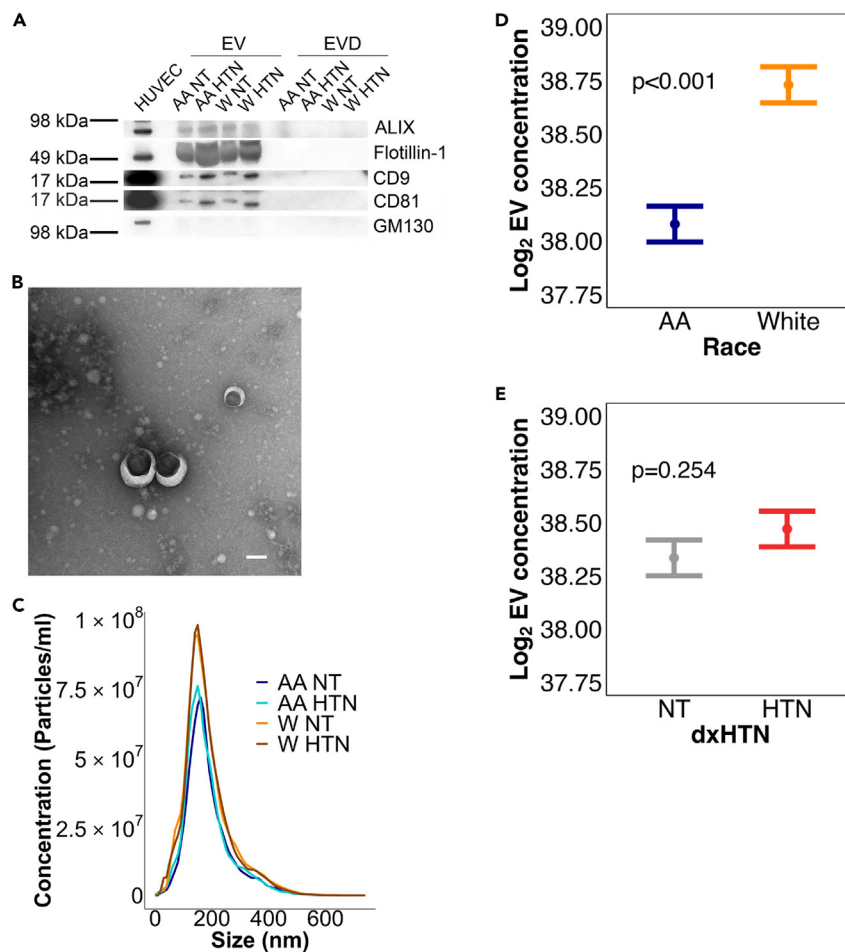
**Figure 1. Characterization of plasma EVs**

Plasma EVs were isolated from participants in this study (Table 1) (n = 215).

(A) Plasma EVs and human umbilical vein endothelial cells (HUVEC) were lysed and immunoblotted with antibodies against known EV markers ALIX, CD9, CD81, and Flotillin-1. GM130 was used as a purity marker.

(B) EVs were isolated, and electron microscopy was used to visualize EV morphology and size (scale bar: 100 nm).

(C) Nanoparticle tracking analysis was used to examine EV size and distribution. The distribution was averaged for each group (AA NT = 54, AA HTN = 54, W NT = 53, W HTN = 54).

(D and E) EV concentration values were $\log_2$ transformed. Linear regression was used to examine the relationship between EV concentration and hypertension, sex, and race (n = 215). The plots show the linear regression values $\pm$ standard error of the estimated values for race and hypertension, respectively.

AA, African American; dxHTN, hypertension status; EV, extracellular vesicle sample; EVD, EV-depleted sample; NT, normotensive; HTN, hypertensive; HUVEC, human umbilical vein endothelial cells; W, White.

sample preparation.[54] Additionally, Nanoparticle Tracking Analysis (NTA) was used to analyze the concentration and size distribution of the EVs (Figure 1C). The size distribution displayed a peak around 195 nm (Figure 1C). Linear regression was used to examine the relationship between EV concentration and hypertension, sex, and race. We found that EV concentration was significantly higher in White participants compared with African American participants (p < 0.001) (Figure 1D) but there were no significant differences in EV concentration with hypertension (p = 0.254) (Figure 1E).

### Plasma mtDNA is associated with race

ccf-mtDNA has been shown to act as a DAMP molecule. Because DAMPs promote sterile inflammatory pathways, we hypothesized that levels of ccf-mtDNA would be higher in the plasma of participants with hypertension. DNA was isolated from the plasma of participants, and levels were analyzed using an established methodology, which we have previously reported.[16,18] Plasma mtDNA values were $\log_2$ transformed, and the relationship between the mtDNA regions was analyzed using Pearson correlation (Figure 2A). All four mtDNA regions were significantly positively correlated with each other, suggesting that all were present in plasma at detectable levels (Figure 2A). We then used linear regression to analyze the relationship between each plasma mtDNA region with hypertension, sex, and race. Plasma mtDNA levels were not
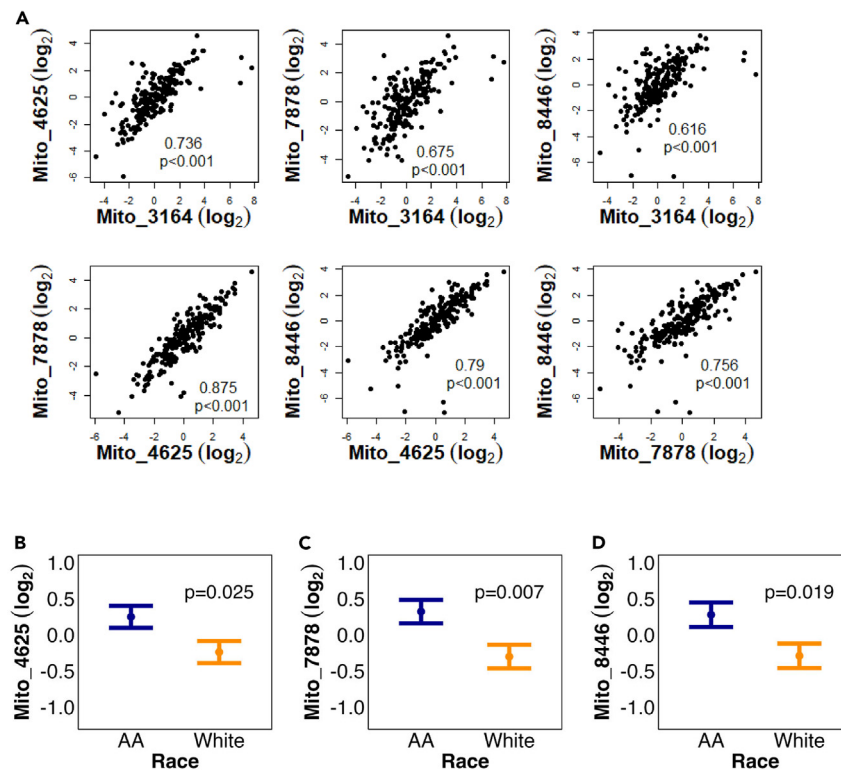
**Figure 2. Plasma mtDNA levels are positively correlated and higher in African Americans**

DNA was isolated from the plasma of participants in this cohort (Table 1). Levels of mtDNA were measured using four unique mtDNA-specific primers.

(A) Plasma mtDNA values were $\log_2$ transformed and analyzed by Pearson correlation. The r and p values are shown for each correlation.

(B–D) Linear regression was used to examine the relationship between plasma mtDNA and hypertension, race, and sex. Plasma mtDNA levels amplified by the (B) Mito_4625 (n = 213), (C) Mito_7878 (n = 214), and (D) Mito_8446 (n = 214) were significantly higher in AA. The plots show the linear regression values ± standard error of the estimated values for (B) Mito_4625, (C) Mito_7878, and (D) Mito_8446.

AA, African American.

significantly associated with hypertension (Mito_3164, p = 0.076; Mito_4625, p = 0.590; Mito_7878, p = 0.882; Mito_8446, p = 0.533). However, three out of the four regions were significantly associated with race: Mito_4625 (p = 0.025), Mito_7878 (p = 0.007), and Mito_8446 (p = 0.019) (Figures 2B–2D). Plasma mtDNA levels were significantly higher in African American participants compared with White participants (Figures 2B–2D).

## EV mtDNA levels are associated with race and mitochondrial DNA haplogroup

DNA was isolated from plasma EVs, and levels of EV mtDNA were measured using quantitative real-time PCR (qPCR). The relationship between the EV mtDNA regions was analyzed using Pearson correlation (Figure 3A). All four mtDNA regions were significantly positively correlated with each other (Figure 3A). This demonstrates that all four regions of the mitochondrial genome were present at detectable levels in the EVs. We then used linear regression to analyze the relationship between each EV mtDNA region and hypertension, sex, and race. For Mito_3164 (p < 0.001), Mito_4625 (p < 0.001), Mito_7878 (p < 0.001), and Mito_8446 (p < 0.001), EV mtDNA levels were higher in African American participants compared with White participants (Figures 3B–3E).

Due to the association of race with levels of plasma and EV mtDNA, we examined how ancestry affected this relationship. We also examined the association between haplogroup and hypertension. Therefore, we haplotyped cohort participants and categorized the resulting haplogroups by ancestry into African, European, and Other (Table 1; Table S1). Participants were grouped into the following mtDNA haplogroups: African (L0, 1, 2, 4, 5, 6, and L3), European (H, HV, J, K, T, U, and UK), and Other (B-P-F-R, M, and N-A-Y-W-I-X) (Table 1). There was concordance between race and mtDNA haplogroup for most participants; 72% (78/108) of the African American participants, 79% (84/107) of White participants, or 75% of all participants (162/215) (Table S2). However, there were some participants whose racial self-identification was different from the determined mtDNA haplogroup. mtDNA haplogroups that were not African or European were designated into the Other category but due to the low participant numbers (n = 12), the Other haplogroup was excluded from all analyses.

We found that there was no association between mtDNA haplogroup and hypertension status in the cohort (Table 1). However, this result may be inconclusive because of the limited size of our cohort. There was a significant interaction between race and mtDNA haplogroup for EV mtDNA levels of the Mito_3164 (p = 0.045), Mito_4625 (p = 0.024), and Mito_8446 (p = 0.022) regions. EV mtDNA levels were significantly
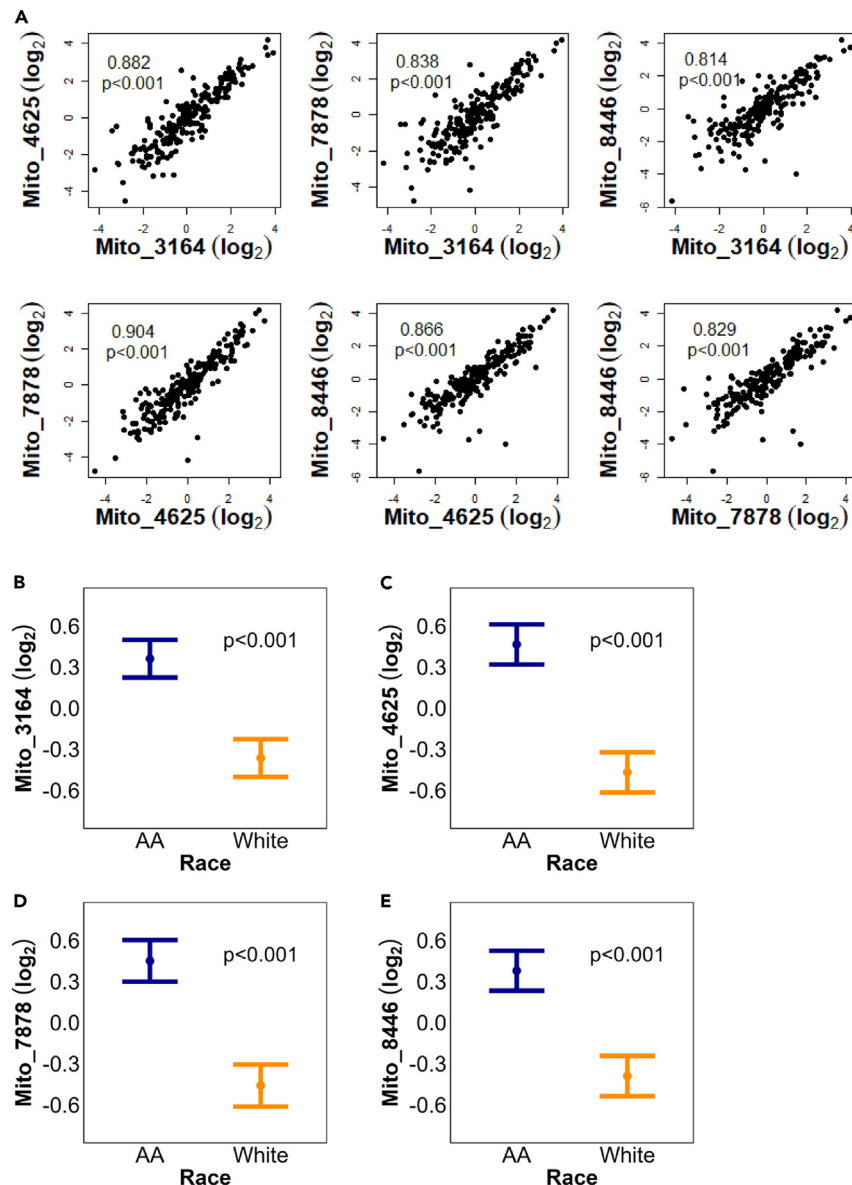
**Figure 3. EV mtDNA levels are positively correlated and higher in African Americans**

Plasma EVs were isolated from the participants in this cohort (Table 1). DNA was then isolated from the EVs, and levels of mtDNA were measured using four mtDNA-specific primers targeting different regions of the mitochondrial genome (n = 215).

(A) EV mtDNA values were $\log_2$ transformed and analyzed by Pearson correlation. The r and p values are shown for each correlation.

(B–E) Linear regression was used to investigate the relationship between EV mtDNA and hypertension, sex, and race. EV mtDNA levels amplified by the (B) Mito_3164 (n = 215), (C) Mito_4625 (n = 215), (D) Mito_7878 (n = 214), and (E) Mito_8446 (n = 214) were significantly higher in AAs. The plots show the linear regression values $\pm$ standard error of the estimated values.

AA, African American.

higher in participants who self-identified as African American with the African haplogroup compared with participants who self-identified as White with the African haplogroup, as well as participants who self-identified as African American with the European haplogroup (Figures 4A– 4C). These data indicate that race and ancestry are associated with EV mtDNA levels. For plasma mtDNA levels, there were no significant interactions between race and mtDNA haplogroup (Mito_3164, p = 0.192; Mito_4625, p = 0.391; Mito_7878, p = 0.104; Mito_8446, p = 0.235).

### Serum inflammatory protein levels are associated with mitochondrial DNA haplogroup, race, and EV mtDNA

ccf-mtDNA can act as a DAMP and elicit signaling pathways that promote inflammation. Therefore, we assessed the relationship between EV mtDNA, race, hypertension status, and mtDNA haplogroup and several circulating inflammatory proteins: interferon gamma (IFN-γ),
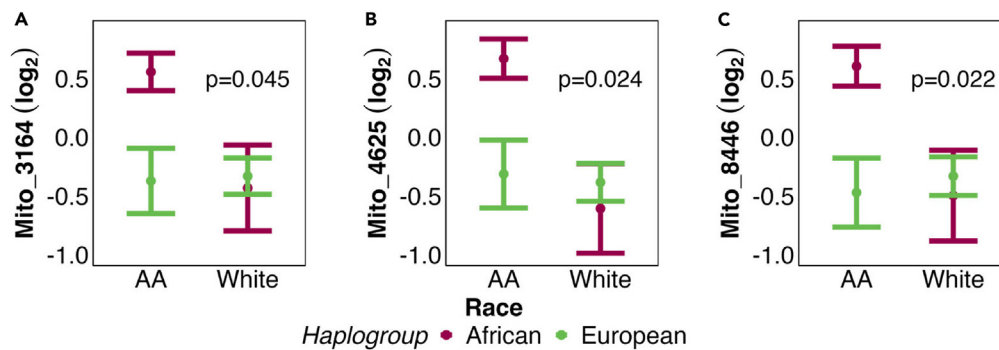
**Figure 4. Higher EV mtDNA in African American individuals with the African haplogroup**

(A–C) Plasma EVs were isolated from the participants of this cohort (Table 1), and DNA was isolated from the EVs. Levels of mtDNA were measured using four mtDNA specific primers targeting different regions of the mitochondrial genome (n = 215). EV mtDNA values were $\log_2$ transformed. Linear regression was used to analyze the relationship between EV mtDNA and mtDNA haplogroup and race. The plots show the linear regression values $\pm$ standard error of the estimated values for (A) Mito_3164 (n = 203), (B) Mito_4625 (n = 203), and (C) Mito_8446 (n = 202). For all three regions, EV mtDNA levels were highest in AA individuals with the African haplogroup.

AA, African American.

interleukin-6 (IL-6), tumor necrosis factor alpha (TNF-$\alpha$) trimer, E-selectin, MCP-1, sRAGE, SAA, P-selectin, and fibrinogen. Two inflammatory proteins, MCP-1 and P-selectin, were significantly associated with race. Overall, MCP-1 and P-selectin serum levels were higher in White participants than African American participants. MCP-1 levels were higher in White participants than African American participants in the models for all four EV mtDNA regions (Mito_3164 p < 0.001; Mito_4625, p < 0.001; Mito_7878, p < 0.001; Mito_8446, p < 0.001) (Figure S1A). P-selectin levels were also significantly higher in White participants in models for three of the EV mtDNA regions (Mito_4625, p = 0.037; Mito_7878, p = 0.044; Mito_8446, p = 0.033) (Figure S1B). In addition, P-selectin levels were significantly related to Mito_4625 (p = 0.027) and Mito_8446 (p = 0.037) regions with higher EV mtDNA levels corresponding to higher P-selectin levels (Figure S1C).

TNF-$\alpha$ trimer levels were significantly higher in participants with the African haplogroup for each of the four EV mtDNA regions (Mito_3164, p = 0.045; Mito_4625, p = 0.044; Mito_7878, p = 0.042; Mito_8446, p = 0.046) (Figure 5A). There was a significant interaction between haplogroup and race for SAA levels in models for each of the EV mtDNA regions (Mito_3164, p = 0.026; Mito_4625, p = 0.009; Mito_7878, p = 0.028; Mito_8446, p = 0.020) (Figure 5B). Levels of SAA were highest in individuals who self-identified as African American with the African haplogroup compared with those who self-identified as African American with the European haplogroup (Figure 5B). SAA was also significantly higher in those who self-identified as White with the European haplogroup compared with those who self-identified as African American with the European haplogroup (Figure 5B).

Models for sRAGE and SAA included a significant interaction between EV mtDNA and haplogroup. Levels of sRAGE for participants with an African haplogroup were positively associated with levels of Mito_3164 (p = 0.011) and Mito_4625 (p = 0.009) compared with those with European haplogroups (Figure 5C). Levels of SAA for participants with an African haplogroup were negatively associated with levels of Mito_4625 (p = 0.042) (Figure 5D).

One inflammatory protein, E-selectin, resulted in models with a significant interaction between race and three of the EV mtDNA regions: Mito_4625 (p = 0.011), Mito_7878 (p = 0.045), and Mito_8446 (p = 0.028) (Figure 5E). High levels of E-selectin for African American participants were associated with lower levels of the EV mtDNA regions, compared with White participants in whom high levels of E-selectin were associated with higher levels of EV mtDNA (Figure 5E).

## DISCUSSION

In the present study, there was no association between plasma ccf-mtDNA, EV mtDNA, or mt haplogroup and hypertension as we had hypothesized. However, we found that EV mtDNA levels were higher in African American participants compared with White participants. We extended this initial finding by determining the role that ancestry, as defined by mtDNA haplogroup, played in examining variation in EV mtDNA levels. We found that EV mtDNA levels were highest in African American individuals with the African haplogroup. Given that ccf-mtDNA can act as a DAMP that may drive inflammatory pathways, we examined inflammatory markers and found that TNF-$\alpha$ trimer, SAA, sRAGE, and E-selectin levels were associated with mtDNA haplogroup, race, and EV mtDNA.

Previous studies have suggested that ccf-mtDNA may be associated with spontaneous hypertension in rats[14], in resistant hypertension in African American men and women[13] as well as in the hypertension-related condition, pre-eclampsia.[55] There is also some evidence that ccf-mtDNA is present in the urine hypertensive African American patients.[56] However, in this study we were unable to identify an association between hypertension and ccf-mtDNA in this middle-aged cohort.

In our analysis, we found that EV mtDNA levels were associated with an interaction of mtDNA haplogroups and race, but we did not find any differences with ccf-mtDNA isolated from whole plasma with haplogroups. These data indicate that there are potentially different mechanisms in the pool of ccf-mtDNA in the circulation. In fact, we are only beginning to understand the mechanisms that result in the release of
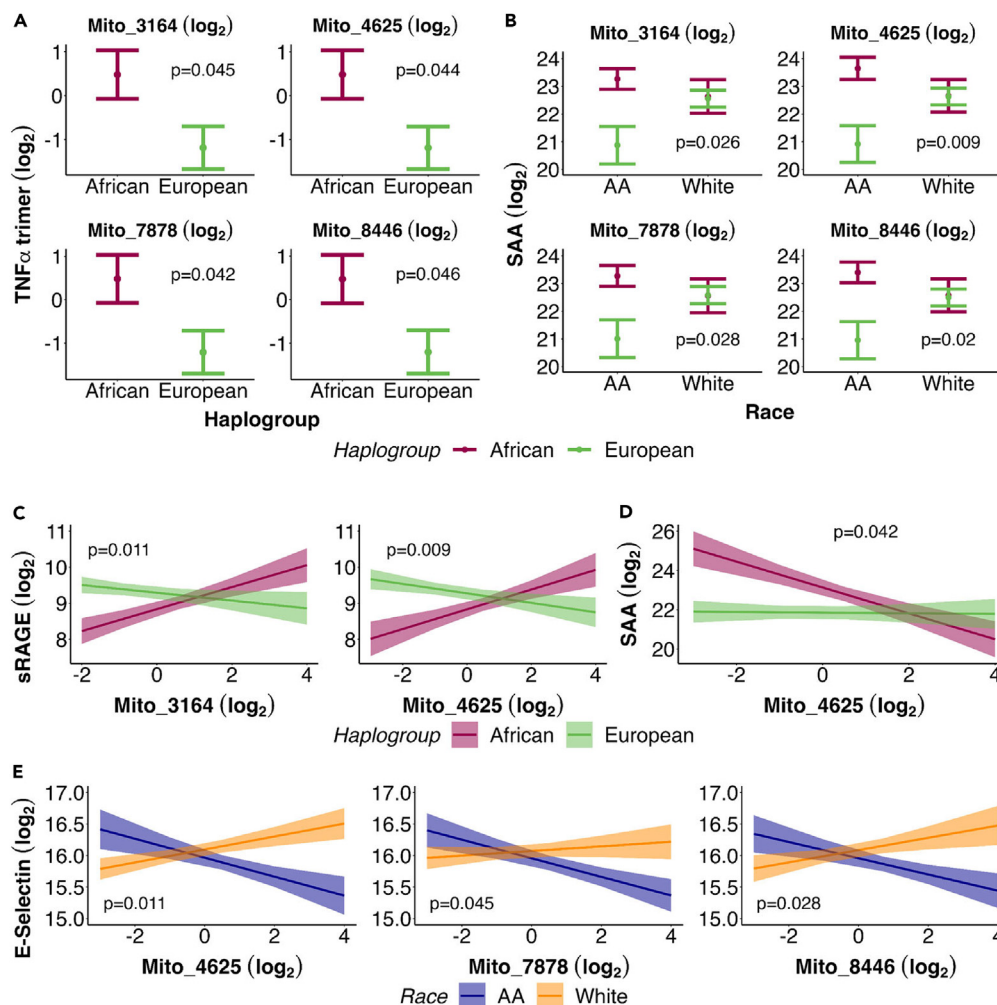
**Figure 5. Inflammatory protein levels are associated with mitochondrial DNA haplogroup, race, and EV mtDNA**

Nine inflammatory proteins were assayed from the serum of HANDLS participants in this cohort (n = 85). Each protein was $\log_2$ transformed for normality, as were the EV mtDNA levels. Linear regression was used to analyze the relationship between each inflammatory protein and hypertension status, race, each EV mtDNA region, and mtDNA haplogroup.

(A) TNF-α trimer levels were significantly different with haplogroup for each EV mtDNA region (Mito_3164 n = 67, Mito_4625 n = 67, Mito_7878 n = 66, Mito_8446 n = 66).

(B) SAA levels were significantly different with haplogroup and race for each EV mtDNA region (Mito_3164 n = 78, Mito_4625 n = 78, Mito_7878 n = 77, Mito_8446 n = 77).

(C) sRAGE were significantly different with haplogroup and EV mtDNA for two regions, Mito_3164 (n = 78) and Mito_4625 (n = 78).

(D) SAA levels were significantly different with EV mtDNA and haplogroup for the Mito_4625 region (n = 78).

(E) E-selectin levels were significantly different with EV mtDNA and race for three regions, Mito_4625 (n = 78), Mito_7878 (n = 77), and Mito_8446 (n = 77). The plots show the linear regression values ± standard error of the estimated values. AA, African American.

ccf-mtDNA in the circulation.[57] These include passive release from dying cells, through either apoptosis or necrosis, or active mechanisms through sorting of mitochondria or mtDNA into extracellular vesicles or naked mitochondria or mtDNA into the circulation.[57] Our data suggest that ccf-mtDNA in plasma versus extracellular vesicles may represent different mechanisms of sorting. It is interesting to speculate that internalization of EVs containing mtDNA would result in release of mtDNA into the cytoplasm and activation of the cGAS-STING pathway that can then promote the release of inflammatory molecules (for review[58]). However, more studies are warranted to investigate the different pools of ccf-mtDNA and how they may function.

We report higher EV mtDNA levels in African Americans with African ancestry compared with European ancestry. It may seem surprising that individuals who identify as African American can have either the African or European haplogroup. However, race is a social construct, whereas mtDNA haplogroup reflects genetic ancestry. mtDNA haplogroups are maternally inherited and are influenced by population migration. Over time this results in a haplogroup of one ethnicity dispersed among another ethnic group that may yield

discordance between race and ancestry. In fact, several other studies examining mtDNA haplogroups and race have also reported discordance of race and ancestry.[59–61] In the National Health and Nutrition Examination Surveys (NHANES), 18,832 participants were classified into mtDNA haplogroups, and the concordance of race with haplogroup varied among the racial/ethnic groups, with non-Hispanic Whites being 94.8% whereas non-Hispanic Blacks were 88.3%.[60] These data indicate the importance of examining both race and ancestry in biomarker studies.

mtDNA haplogroups have been associated with an altered risk of CVD in some studies; however, the subject is complicated by study differences in which subsets of populations and haplogroups are analyzed. mtDNA polymorphisms considered non-pathogenic, such as the European haplogroups H and T, have been linked with an increased risk of CVD compared with other European haplogroups.[49] In a knee osteoarthritis study on White participants with European haplogroups, participants with the J haplogroup displayed a lower risk of CVD compared with other common European haplogroups.[48] Similarly, a Spanish study on risk of ischemic cardiomyopathy (IC) and European haplogroups found that haplogroup J was significantly lower in IC patients compared with healthy controls, whereas haplogroup H was significantly higher in IC patients compared with healthy controls.[62] However, a study on a UK population found few associations between European haplogroups and transient ischemic attack or ischemic stroke, aside from a lower risk in participants with haplogroup K.[63] Calabrese et al. found no difference in the frequency of European haplogroups between participants with hypertension, ischemic heart disease, ischemic stroke, or healthy controls.[64] Fewer studies include analysis of CVD risk in cohorts containing participants with African mtDNA haplogroups. In a Brazilian study, Cardena et al. found that African mtDNA haplogroups were associated with a diagnosis of hypertensive cardiomyopathy when compared with European haplogroups.[51]

In this study, individuals with an African mtDNA haplogroup who self-identified as African American displayed the highest EV mtDNA levels. Previous research has shown that EV mtDNA levels increase in the plasma of patients with chronic heart failure compared with healthy controls.[65] Other studies have focused on the molecular differences between cells from individuals with varying mtDNA haplogroups, although comparisons utilizing African haplogroups are limited. In a study comparing mitochondrial function and damage in cells from participants with either African or European mtDNA ancestry, cells with haplogroup L displayed more efficient ATP production but also more mtDNA damage than cells with haplogroup H.[44] A study investigating the differences between retinal epithelial cell cytoplasmic hybrids (cybrids) with two European mtDNA haplogroups, J and H, showed that EVs from J-cybrids contained higher levels of fibronectin and annexin A2 compared with H-cybrid EVs, suggesting that J-cybrids were more stressed.[66] To the best of our knowledge, this is the first study to look at ccf-mtDNA levels in the context of mtDNA haplogroups and race.

Here, in addition to examining ccf-mtDNA, we also quantified the levels of circulating inflammatory proteins. These proteins were chosen based on their previous association with social determinants of health, age, or mortality.[67] TNF-α trimer levels were higher in those with the African haplogroup for each of all four EV mtDNA regions. SAA levels were significantly different with both haplogroup and race for all EV mtDNA regions and were highest in individuals who self-identified as African American with the African haplogroup. SAA and sRAGE had a significant interaction between EV mtDNA and haplogroup, whereas E-selectin levels were negatively associated with one EV mtDNA region in African American participants and positively associated with one EV mtDNA region in White participants. These findings are important given that little is known about the relationship between inflammatory markers and the African and European mtDNA haplogroups. A study utilizing retinal pigment epithelia (RPE) cell cybrids with the African (L) or European (H) haplogroup reported that these haplogroups showed differences in nuclear gene expression in pathways related to complement activation, inflammation, and autoimmunity.[43] This same group followed-up this initial study and recently reported that cybrids with the African and European haplogroup have different responses, including inflammatory pathways, to the stressors, amyloid-β, and sublethal ultraviolet radiation doses.[68] These studies indicate that mtDNA haplogroups can modulate gene expression, which can potentially lead to different responses to stress and perhaps susceptibility to specific diseases.

Our study is an initial step into important investigations of the role of ccf-mtDNA as DAMPS that may contribute to the weathering associated with health disparities among populations at risk. This work begins to examine the role of ancestry, not just the social construct of race, as risk factors for the development of age-related chronic disease. These findings contribute to the extant literature on the interplay between race, mtDNA haplogroup, and circulating factors, including EVs and their cargo and inflammatory markers. Future advances in understanding the drivers of health disparities may need to consider these and other complex factors to understand the biological mechanisms through which the social determinants of health lead to disparate health outcomes.

## Limitations of the study

Findings from this study should be considered within the context of its limitations. The sample size for this study is aligned with many EV-based studies.[16,18,24,69] However, it may be limited to assess the association of mtDNA haplogroup with hypertension. The age of the cohort is both a strength and limitation to the study. At middle-age, perhaps the cohort is too young to uncover associations of EVs and their cargo with hypertension and with mtDNA haplogroup and hypertension. However, middle-age is a critical period for African Americans who develop hypertension earlier in the life span, which is concordant with the theory of accelerated aging. Examining middle-aged cohorts is particularly important because hypertension is a well-established preventable driver of CVD and a risk factor for morbidity and mortality.

Several other strengths of our study consist of the inclusion of both African American and White adults, with the addition of defining genetic ancestry through mtDNA haplogroup. Few studies have included racially diverse cohorts in EV-based studies.[24] In addition, even in larger epidemiological studies of mtDNA haplogroup, the focus has been European mtDNA haplogroups (H, J, K, etc.).

## STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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## SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2023.108724.

## AUTHOR CONTRIBUTIONS

A.B.Z., N.M., N.N.H., and M.K.E. conceived and designed the study. A.M.B. and V.O. executed the experiments. A.M.B., V.O., and J.T.S. performed data collection. A.M.B. and V.O. performed statistical analysis and generated figures with help from N.M. and A.B.Z. N.E. and M.K.E. performed the medical evaluation of participants and supervised collection of biomaterials. A.B.Z. and M.K.E. are co-principal investigators for HANDLS. A.M.B., N.N.H., and J.T.S. wrote the manuscript with input from all the authors. All authors read and approved the final manuscript.

## DECLARATION OF INTERESTS

The authors declare no conflicts of interest.

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# STAR★METHODS

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| **Antibodies** | | |
| ALIX | Santa Cruz Biotechnology | Cat# sc-271975; RRID: AB_10707811 |
| CD9 | System Biosciences | Cat# EXOAB-CD9A-1; RRID: AB_2687469 |
| CD81 | System Biosciences | Cat# EXOAB-CD81A-1; RRID: AB_2819191 |
| Flotillin-1 | Abcam | Cat# ab133497; RRID: AB_11156367 |
| GM130 | Abcam | Cat# ab52649; RRID: AB_880266 |
| **Chemicals, peptides, and recombinant proteins** | | |
| Thromboplastin DS | Fisher Scientific | Cat# 100354TS |
| protease inhibitor cocktail | Roche | Cat# 11836170001 |
| Phosphatase inhibitor cocktail | Roche | Cat# 04906837001 |
| ExoQuick™ Exosome Precipitation Solution | System Biosciences | Cat# EXOQ20A-1 |
| Mammalian Protein Extraction Reagent (MPER®) | Thermo Scientific | Cat# 78501 |
| 400 mesh formvar/carbon coated grids | Electron Microscopy Sciences | Cat# CF400-Cu-UL |
| 4-12% NuPAGE Bis-Tris gel | ThermoFisher Scientific, | Cat# NW04125BOX |
| NuPAGE™ MES SDS Running Buffer | ThermoFisher Scientific, | Cat# P002 |
| Tween® 20 Detergent | Sigma | Cat# P1379-100ML |
| KwikQuant Ultra HRP Substrate Solution | Kindle Biosciences, LLC | Cat# R1002 |
| Baseline-ZERO DNase | Lucigen | Cat# DB0715K |
| TaqMan™ Fast Advanced Master Mix | Applied Biosystems | Cat# 4444557 |
| TaqPath™ ProAmp™ Master Mix | Applied Biosystems | Cat# A30865 |
| **Critical commercial assays** | | |
| DNeasy Blood and Tissue kit | Qiagen | Cat# 69506 |
| **Experimental models: Cell lines** | | |
| Human umbilical vein endothelial cell | Lonza | Cat# CC-2519 |
| **Oligonucleotides** | | |
| Mito_3164: F and R primers: 5′CCTTCCCCCGT AAATGATATCA3′/5′GCCATCTTAACAAACCCTGTTCTT3′ Probe: 5′FAM-AACTTAGTATTATACCCACACCC-MGB3′ | Applied Biosystems | Custom |
| Mito_4625: F and R primers: 5′CACAGAAGCTGCCAT CAAGTA3′/5′CCGGAGAGTATATTGTTGAAGAG3′ Probe: 5′FAM-CCTCACGCAAGCAACCGCATCC-BLACKHOLE-3′ | Applied Biosystems | Custom |
| Mito_7878: F and R primers: 5′AATCAAT TGGCGACCAATGG3′/5′CGCCTGGTTCT AGGAATAATGG3′ Probe: 5′FAM-ACTG AACCTACGAGTACAC-MGB-3′ | Applied Biosystems | Custom |
| Mito_8446: F and R primers: 5′AATATTAAACACAAACTAC CACCTACCT3′/5′TGGTTCTCAGGGGTTTGTTATAA3′ Probe: 5′-FAM-CCTCACCAAAGCCCATA-MGB-3′ | Applied Biosystems | Custom |
| **Software and algorithms** | | |
| R | R Core Team | https://www.R-project.org |
| NTA 3.4 Build 3.4.4 | Malvern Panalytical | https://www.malvernpanalytical.com/en/ |

*Continued*

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| Applied Biosystems, software version SDS 2.4.1 | Applied Biosystems | https://www.thermofisher.com/us/en/home/brands/applied-biosystems.html |
| QuantStudio software | Applied Biosystems | https://www.thermofisher.com/us/en/home/brands/applied-biosystems.html |
| TaqMan Genotyper software | Applied Biosystems | https://www.thermofisher.com/us/en/home/brands/applied-biosystems.html |
| Other | | |
| SP-X Multiplex Planar Immunoassay | Quanterix | Custom services |
| Determination of mitochondrial DNA haplogroup using allelic discrimination primers | Department of Ophthalmology Research, University of California Irvine | Custom services |

## RESOURCE AVAILABILITY

### Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Michele K. Evans (me42v@nih.gov).

### Materials availability

This study did not generate new unique reagents.

### Data and code availability

- The data reported in this study cannot be deposited in a public repository because of ethical restrictions imposed by the National Institutes of Health, Institutional Review Board (IRB). The IRB approved consent forms that our study participants signed do not permit unrestricted public data sharing. However, it does permit data sharing through a Data Use Sharing Agreement which protect the privacy of human research participants and the confidentiality of their data. To request access see the HANDLS website (https://handls.nih.gov/06Coll.htm) and contact Ms. Jennifer Norbeck, MSW, CCRC (norbeckje@grc.nia.nih.gov) 410-558-8622).
- This paper does not report original code. However, the R script used to produce the statistical analyses used in the paper has been made publicly available on the HANDLS website (https://handls.nih.gov/Rscripts/2024-Byappanahalli_iScience.R).
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

## EXPERIMENTAL MODEL AND STUDY PARTICIPANTS DETAILS

### Human participants

A subcohort was selected from the Healthy Aging in Neighborhoods of Diversity Across the Life Span (HANDLS) study of the National Institute on Aging Intramural Research Program, National Institutes of Health (NIH).[70] The Institutional Review Board of the National Institutes of Health approved the HANDLS study and all participants provided written informed consent. HANDLS is a longitudinal study of community-dwelling non-Hispanic African American and White adults in Baltimore, Maryland, which examines the role of social and biological factors in age-related health disparities and in the development of age-associated illnesses. HANDLS started in 2004. Since then, participants have been examined approximately every 5 years. Race was self-reported as either African American or White, sex was sex assigned at birth, and poverty status (living above or below poverty) was based on household income at enrollment as defined by 125% of the 2004 U.S. Health and Human Services Poverty Guidelines.[71] Standardized blood pressure measurements were taken by medical staff and the mean of up to two measurements were used for each participant. Individuals categorized as hypertensive met at least one of the following criteria: systolic blood pressure $\geq 140$ mmHg, diastolic blood pressure $\geq 90$ mmHg, self-reported previous diagnosis by health care provider, or current prescribed medication for hypertension.

For this study, we used a balanced factorial design across race, sex, and hypertensive status and randomly selected 27 people for each condition, or 216 participants total, with a blood sample from HANDLS wave 4 (2013–2017) aged 45–59 years. Sample size was based on our ability to detect differences in similar cohorts.[18,69,72] The initial subcohort consisted of 108 normotensive and 108 hypertensive participants but due to plasma sample availability, the final subcohort consisted of 107 normotensive and 108 hypertensive participants (Table 1).

## METHOD DETAILS

### Blood samples

Blood samples were collected in the morning following overnight fasting into sodium heparin blood collection tubes. To isolate plasma, Histopaque-1077 (Sigma-Aldrich Cat: 10771) was slowly added to the blood samples in 15 mL conical tubes. Samples were then centrifuged for 20 min at 610 $g$ with a slow deceleration. After successful separation, the top plasma layer was aliquoted and stored at $-80°C$.

### Plasma EV isolation

We isolated EVs blinded by group assignment from plasma using a previously established protocol.[69] Briefly, plasma (0.45 mL) was thawed on ice, treated with 0.15 mL Thromboplastin DS (Fisher Scientific, Inc. Cat:100354TS), and incubated at room temperature for 30 min. To separate fibrin proteins, 0.4 mL of phosphate buffered saline (PBS) was supplemented with 3X concentrated protease (Roche, Cat: 11836170001) and phosphatase (Roche, Cat: 04906837001) inhibitor cocktails, mixed by inversion, and centrifuged at 3,000 $g$ for 20 min at 4°C. The supernatant was collected and 252 µL of ExoQuick Exosome Precipitation Solution (System Biosciences, Cat: EXOQ20A-1) was added per sample, incubated for 1 h at 4°C, and then centrifuged at 1,500 $g$ for 20 min at 4°C. The supernatant was collected for subsequent analysis as the EV-depleted (EVD) fraction. The pellet containing EVs was resuspended in 0.5 mL PBS supplemented with 3X concentrated protease and phosphatase inhibitor cocktails as described above. Samples were aliquoted (50 µL) for DNA isolation, diluted (1:300) in filtered PBS for NTA analysis, and lysed in Mammalian Protein Extraction Reagent (MPER, Thermo Scientific, Cat: 78501) prior to storage at $-80°C$ for future analysis.

### Electron microscopy

Electron microscopy was performed by the Johns Hopkins University School of Medicine Microscope Facility as previously reported in.[18] Briefly, plasma-derived EVs were isolated and absorbed to freshly ionized 400 mesh formvar/carbon coated grids (Electron Microscopy Sciences, Cat: CF400-Cu-UL). Samples were then washed with Tris-buffered saline (TBS, 3 drops) and negatively stained in 1% aqueous uranyl acetate. Images were then taken with a transmission electron microscope (Hitachi 7600 TEM) at 120 kV using an 8mP 16bit Dual AMT CCD camera system.

### Nanoparticle tracking analysis

EV size distribution and concentration were analyzed using nanoparticle tracking analysis (NTA) on a NanoSight NS500 (Malvern Panalytical, software version NTA 3.4 Build 3.4.4). Isolated EV samples were initially diluted 1:300 in filtered PBS. Some samples were further diluted due to variation in concentration. EV samples were recorded in five videos of 20 s at camera level 16 and detection level 6. For measurement accuracy, all samples were analyzed on the same instrument and by a single operator. Total plasma EV concentration was calculated, accounting for final dilution factor, starting plasma and final EV sample volume, as previously described.[69] EV concentration values were $\log_2$ transformed as they were positively skewed.

### Immunoblotting

EVs were lysed in MPER supplemented with 3X concentrated protease and phosphatase inhibitor cocktails as described above. Protein samples of 2.5 µg for EV and EV-depleted (EVD) samples were run on a 4–12% NuPAGE Bis-Tris gel under sodium dodecyl sulfate (SDS)-denaturing conditions (ThermoFisher Scientific, Cat: NW04125BOX) with 1X NuPAGE MES SDS Running Buffer (ThermoFisher Scientific, Cat: NP002) for higher resolution of low molecular weight proteins. Human umbilical vein endothelial cell (HUVEC) lysate was used as a positive control (3.7 µg). The gel was transferred onto polyvinylidene difluoride (PVDF) membrane. After blocking in 3% BSA in TBS with 0.1% Tween 20 Detergent (Sigma, P1379-100 ML), the membrane was incubated with primary antibodies for 1 h at room temperature: ALIX (Santa Cruz Biotechnology, Cat: SC-271975), CD9 (System Biosciences, Cat: EXOAB-CD9A-1), CD81 (System Biosciences, Cat: EXOAB-CD81A-1), Flotillin-1 (Abcam, Cat: ab133497), and GM130 (Abcam, Cat: ab52649). All primary antibodies were diluted 1:500. For detection, the membranes were incubated with the appropriate secondary horseradish peroxidase (HRP)-conjugated antibodies at 1:5,000 for anti-rabbit and anti-mouse for 32 min. These blots were visualized with the KwikQuant Ultra HRP Substrate Solution and imaging system according to the manufacturer's protocol (Kindle Biosciences, LLC; Cat: R1002).

### DNA isolation from plasma-derived EVs and plasma

Previously, we established an experimental protocol for analyzing EV mtDNA levels.[16,18] EV aliquots (50 µL) were thawed and treated with DNase to degrade surface or non-vesicular DNA using 5 U of DNase (Lucigen, Baseline-ZERO DNase, Cat: DB0715K) for 30 min at 37°C. The reaction was stopped by adding 6.5 µL DNase Stop Solution for 10 min at 65°C. Then, the EV volume was equalized to 200 µL with the addition of 128 µL of nuclease-free water. DNA was then isolated using the DNeasy Blood and Tissue kit (Qiagen, Cat: 69506). An additional spin was conducted at 20,000 $g$ for 1 min after adding Buffer AW2. Fresh waste collection tubes were used in between each spin. A 5 min incubation at room temperature of 50 µL AW buffer in the spin column was added before the final 1 min 8,000 $g$ spin for DNA elution. The eluted DNA (~50 µL) was further diluted in an additional 50 µL AE Buffer (Qiagen) and stored at $-20°C$.

Plasma samples (45 µL) were thawed on ice and nuclease-free water (155 µL) was added to reach a final volume of 200 µL. DNA was isolated as described above using the DNeasy Blood and Tissue kit with the exception that the samples were not DNase treated.

### Quantitative real-time PCR

Quantitative real-time PCR (qPCR) was performed as previously reported in[16,18] for both EV and plasma DNA. Four different primer sets targeting different regions of the mitochondrial genome were used[16,18]: Mito_3164 for the region between the mitochondrially encoded 16S rRNA (*MT-RNR2*) and mitochondrially encoded tRNA leucine 1 (UUA/G) (*MT-TL1*) genes, Mito_4625 for the mitochondrially encoded NADH dehydrogenase 2 (*MT-ND2*) gene region, Mito_7878 for the mitochondrially encoded cytochrome *c* oxidase subunit II (*MT-CO2*) (*MT-CO2*) gene region, and Mito_8446 for the mitochondrially encoded ATP synthase 8 (*MT-ATP8*) gene region. Briefly, the total volume of each reaction was 13 μL, including mitochondrial gene-specific primers (2.5 μL/reaction), TaqMan Fast Advanced Master Mix (7.5 μL/reaction, Applied Biosystems, Cat: 4444557), and isolated DNA (3 μL/reaction).[16,18] A 7900HT Fast Real-Time PCR System was used to run the samples (Applied Biosystems, software version SDS 2.4.1). The following thermal profile was used: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 15 s at 95°C, and 1 min at 60°C. mtDNA levels were calculated using a previously established analysis pipeline.[16,18] EV and plasma mtDNA values for each primer set were positively skewed and were $\log_2$ transformed for analysis. A quality check was conducted to remove outlier values that were five standard deviations from the mean. No outliers were detected in the four EV mtDNA regions. One outlier was detected in plasma for the Mito_3164 region. This sample was removed from all four plasma mtDNA regions and excluded in further analyses.

### Determination of mitochondrial DNA haplogroups

mtDNA haplogroups were determined via allelic discrimination PCR assays performed by the Department of Ophthalmology Research, University of California Irvine.[73] Ten allelic discrimination primers were synthesized by ABI Assay-by-Design (Applied Biosystems). Primers were chosen to give the greatest coverage in European and African mtDNA haplogroups. These primers do not overlap and are >100 base pairs from the mtDNA primers used for qPCR. Samples were run with TaqPath ProAmp Master Mix on a QuantStudio 5 system (Applied Biosystems). Data were analyzed with QuantStudio software, TaqMan Genotyper software, and Microsoft Excel. Data were compared with a simplified map of mtDNA lineages from www.mitomap.org. Participants were sorted into the following mtDNA haplogroups or macrohaplogroups: L0,1,2,4,5,6; L3; H; HV; J; K; T; U; UK; B-P-F-R; M; and N-A-Y-W-I-X. Participants were then grouped into African, European, or Other mtDNA haplogroup ancestries. L0,1,2,4,5,6 and L3 were grouped into the African mtDNA ancestry group; H, HV, J, K, T, U, and UK were grouped into the European mtDNA ancestry group; and B-P-F-R, M, and N-A-Y-W-I-X were grouped into the Other mtDNA ancestry group.

### Inflammatory protein measurements

Nine different serum inflammatory-related proteins (IFNγ, IL-6, TNFα trimer, E-Selectin, MCP-1, soluble receptor for advanced glycation end-products (sRAGE), serum amyloid A (SAA), P-Selectin, fibrinogen) were assayed using an SP-X Multiplex Planar Immunoassay on the Quanterix SP-X platform (Billerica, MA, USA). These proteins were analyzed based on their probable roles as inflammatory markers in age-associated diseases and conditions.[67] Values outside the limit of detection were removed. All inflammatory proteins had <1% outside the limit of detection with the exception of IFNγ (2.6%) and TNFα trimer (15.9%). All the inflammatory protein values were positively skewed. Thus, the values were $\log_2$ transformed for statistical analysis.

### QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analyses were performed using R software version 4.2.0.[74] Student's *t* test was used to analyze differences between groups for age, as well as systolic and diastolic blood pressure. Pearson's Chi-squared test was used to test for differences across race, sex, poverty status, hypertension medication, and mtDNA haplogroup. Correlations between the four EV mtDNA regions and plasma mtDNA regions were assessed using Pearson correlation (pairwise complete observation). Backward stepwise linear regression was employed whereby all possible interactions up to three-way were considered (except for models examining the two-way interaction of mtDNA haplogroups and race on levels of EV and plasma mtDNA). Non-significant interactions were removed. Models examining EV concentration, as well as EV and plasma mtDNA levels, were modeled to the study design of hypertension, race, and sex. Models examining mtDNA haplogroup status on levels of EV and plasma mtDNA included only mtDNA haplogroup and race in the model. The linear regression models examining each inflammatory protein included hypertension status, haplogroup, race, and each EV mtDNA region; therefore, there were four models per inflammatory protein. A p value <0.05 was used as a threshold for statistical significance. Statistical tests performed, sample sizes and description of the values shown are noted in the table and figure captions.