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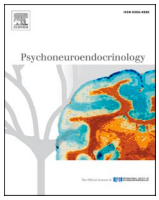

Association of psychosocial stress and poverty with plasma and extracellular vesicle mitochondrial DNA levels

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ABSTRACT

Perceived discrimination has been associated with poor age-related health outcomes, yet little is known about the molecular mechanisms. Previous data have indicated that exposure to acute psychological stress results in higher levels of circulating cell-free mitochondrial DNA (ccf-DNA) in blood. ccf-mtDNA is part of the damage-associated molecular pattern (DAMP) system where it can activate the innate immune system. The current study examined the relationship between perceived discrimination, race, and poverty with ccf-mtDNA in plasma and plasma-derived extracellular vesicles (EVs) in a cohort of African American and White men and women who experienced high or low perceived discrimination (N = 64). Ccf-mtDNA levels were quantified from plasma and plasma EVs. We found that among individuals living below poverty, those with higher perceived discrimination had lower ccf-mtDNA levels in both plasma and EVs than those with low perceived discrimination. With advancing age, ccf-mtDNA levels were higher in African American participants, while the opposite was observed in White participants. We also discovered that the EV inflammatory proteins IL-18 and Cystatin-D were associated with perceived discrimination and age, sex, or race. The presence of EV Caspase-8 and TNF were associated with perceived discrimination and poverty, while EV IL-8 and TNF and TWEAK were associated with perceived discrimination and sex. Our findings suggest that discrimination interacts with sex, race, and poverty to influence ccf-mtDNA levels in plasma and EVs and EV inflammatory proteins.

1. Introduction

Psychosocial stress is associated with adverse health outcomes including both psychological and physiological. Negative social experiences are known to "get under the skin" and promote biological processes that can lead to poor health outcomes and the accelerated aging phenotype (Goosby, Cheadle and Mitchell, 2018; Williams et al., 2019). Discrimination is a psychosocial stressor that is defined as the prejudice and unfair treatment of a category of people and is multidimensional in nature and includes experiences on the individual level or by society (Williams and Mohammed, 2009). Increasing evidence indicates that discrimination is associated with a broad range of negative health outcomes (Goosby, Cheadle and Mitchell, 2018; Williams et al., 2019) including metabolic syndrome and diabetes (Beatty Moody et al., 2018; Whitaker et al., 2017), cognition (Barnes et al., 2012; Zahodne, Sol and Kraal, 2017) and brain health (Beatty Moody et al., 2019). Higher levels of inflammation have also been associated with discrimination (reviewed in (Agbonlahor et al., 2024)) and chronic inflammation is an established driver of age-related disease (Howcroft et al., 2013). Therefore, it is important to understand the biological mechanisms that drive the effects of discrimination on health.

Accumulating data indicate that mitochondria are not only energy producing organelles but also sense, integrate, and respond to stress (Picard and McEwen, 2018a). Mitochondria are key stress mediators that relay cellular signals as part of the adaptive stress response. Psychosocial stress has been associated with changes in mitochondrial allostatic load, which may lead to detrimental downstream effects on health (Picard and McEwen, 2018a). However, we are only beginning to

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understand how the mitochondria integrate and adapt to stress, and most studies on acute and chronic stress are in male rodent models (Picard and McEwen, 2018b). Mitochondria contains its own genome and mitochondrial DNA (mtDNA) can be released into the extracellular environment as circulating cell-free mtDNA (ccf-mtDNA). Ccf-mtDNA can be detected in body fluids including plasma and serum. Ccf-mtDNA levels are higher in inflammatory conditions, cancer, myocardial infarction, and sepsis (Boyapati et al., 2017; Trumpff et al., 2021). In the context of stress, studies thus far have focused primarily on ccf-mtDNA under conditions of psychopathological conditions or acute stress (Hummel et al., 2018; Lindqvist et al., 2018; Trumpff et al., 2019, 2021). Therefore, there is a knowledge gap as to whether psychosocial stress, and specifically discrimination, can alter ccf-mtDNA levels.

In conditions of mitochondrial stress or damage, ccf-mtDNA is released as part of the damage-associated molecular pattern (DAMP) system where it can activate the innate immune system. This fraction of ccf-mtDNA is not-membrane bound and therefore accessible to receptors. In the circulation, ccf-mtDNA has been reported to be in the liquid portion of blood and also encapsulated in whole mitochondria (Al Amir Dache et al., 2020; Song et al., 2020; Stephens et al., 2020) or in extracellular vesicles (EVs) (Lazo et al., 2021; Sansone et al., 2017). EVs are small membrane-bound vesicles that are released into the extracellular space, including body fluids, where they can act as communicators between cells and tissues (van Niel et al., 2022). In addition to ccf-mtDNA, EVs also carry other types of cargo including proteins, DNA, RNA and lipids (Dixson et al., 2023; Yanez-Mo et al., 2015). We recently reported that EV mtDNA levels are higher in frail individuals and in African American individuals with the African mtDNA haplogroup (Byappanahalli et al., 2023, 2024). There is substantial interest in utilizing EVs as biomarkers of various diseases (Kalluri and LeBleu, 2020; Noren Hooten and Evans, 2020; Yanez-Mo et al., 2015) and also in mental disorders (Kong et al., 2023). However, little is known about EVs and their cargo in response to psychosocial stress.

In this study, we examine whether perceived discrimination, also referred to as interpersonal or individual level discrimination, is associated with ccf-mtDNA in plasma and EVs in an urban cohort of African American and White middle-aged males and females living above and below the 125 % poverty line who reported low or high perceived discrimination. We framed this study using an intersectional framework because of the clear indications from various perspectives in the literature that individuals experience environmental factors in the context of their social identities and positions (Bowleg, 2012; Routen et al., 2023). Intersectionality goes hand in hand with the concept of the exposome that takes into account the cumulative environmental exposures that contribute to disease, aging, morbidity, and mortality (Argentieri et al., 2025; Wild, 2005). Use of an intersectional framework is also critical to examine differences in health outcomes that may be related to the exposome and the multiple intersecting social identities of individuals (Simkus, Holtz and Twombly, 2024). Previous work has demonstrated the importance of studying the intersectional identities race, sex, and poverty in the context of blood-based biomarkers and health outcomes (Mainous et al., 2024; Noren Hooten et al., 2023, 2022b; Singh and Newman, 2011). Here we hypothesize that psychosocial stress may interact with race, sex, and poverty status to alter levels of ccf-mtDNA in plasma and in EVs. As few studies include both African American and White individuals or use an intersectional framework to explore biological questions, our study will provide novel insight into biological pathways that may underly identity differences in the effects of discrimination.

2. Materials and methods

2.1. Clinical Study Participants

The participants in this study were selected from the HANDLS study by the National Institute on Aging Intramural Research Program at the National Institutes of Health (Evans et al., 2010). The HANDLS study was designed to investigate how race and socioeconomic status influence aging and age-related chronic diseases in middle-aged adults living in Baltimore, Maryland. Race was self-identified as either African American or White. Poverty status was defined as living above or below 125 % of the 2004 US Department of Health and Human Services, (2004) based on household size and income at enrollment. HANDLS received approval by the Institutional Review Board of the National Institutes of Health and all participants provided written informed consent. Participants completed various questionnaires and submitted a blood sample during the first wave of HANDLS data collection (2004–2009). Details on blood collection and processing are in Supplementary Methods according to MIBlood-EV guidelines (Supplementary Methods) (Lucien et al., 2023). In brief, blood was collected in the morning using a 20- or 22-gauge needle into EDTA-containing blood collection tubes, and then centrifuged in a Beckman Coulter Allegra X-14 Centrifuge at 1460 *g* for 15 min at room temperature with the brake on. Plasma was collected, aliquoted, and frozen at −80°C until use. For this study, individuals aged 45–64 years were eligible and those with HIV/AIDS or hepatitis B/C diagnoses were excluded. The final study cohort (n = 64) was determined using a factorial design by sex, race (African American or White), and perceived discrimination levels (high or low) (Table 1). Due to sample availability, one high perceived discrimination sample was replaced for the plasma mtDNA analysis.

Clinical laboratory markers including high-sensitivity C-reactive protein (hsCRP), serum uric acid, and erythrocyte sedimentation rate were measured by Quest Diagnostics (Nichols Institute, Chantilly, VA). Serum growth differentiation factor 15 (GDF15) levels were quantified using Quantikine ELISA kits from R&D systems (Cat#SGD150) as described previously (Freeman et al., 2020). hsCRP values were positively skewed and thus $\log_2$ transformed for analysis.

2.2. Perceived Discrimination

An overall perceived discrimination level was found by combining two assessments: racial discrimination and lifetime discrimination. Lifetime discrimination was assessed via a two-item questionnaire asking if discrimination interfered with the participant's life and if life was harder due to discrimination. The participants could choose from four responses: "not at all," "a little," "some," and "a lot," assigning scores of 1–4 respectfully (Beatty Moody et al., 2019). Racial discrimination was assessed via a six-item questionnaire inquiring if participants experienced discrimination due to their race or ethnicity at school, work, from the police/court, when obtaining employment, when obtaining housing, and when obtaining medical care. Responses of either "no" or "yes" were assigned a score of 1 or 2 (Krieger, 1990). The total score for both assessments were summed with a possible score of 2–8 for lifetime discrimination and 6–12 for racial discrimination. Low overall perceived discrimination was classified as having minimal scores

Table 1
Demographics for study cohort (N = 64).

	Low Perceived Discrimination (N = 32)	High Perceived Discrimination* (N = 32)	p-value[1]
Men, N (%)	16 (50)	16 (50)	1.000
African American, N (%)	16 (50)	16 (50)	1.000
Below Poverty, N (%)	8 (25)	10 (31)	0.781
Age, mean (SD)	55.7 (5.6)	53.3 (5.8)	0.100

* For the plasma mtDNA samples, one high perceived discrimination sample was replaced resulting in an average age of 53.7 and p-value of 0.164; and 9 people living below poverty status for a p-value of 1.000.

on both questionnaires; 2 for lifetime discrimination questionnaire and 6 for racial discrimination. High overall perceived discrimination was classified as a score of 5 or above for lifetime discrimination and 9 or above for racial discrimination.

2.3. DNA isolation from plasma

We followed a previously established protocol to isolate DNA from plasma (Byappanahalli et al., 2024). Plasma samples were thawed and aliquots of 40 µl were added to 160 µl of Nuclease free H_2O to reach a final volume of 200 µl. Using the DNeasy Blood and Tissue kit, DNA was isolated from plasma using the DNeasy Blood and Tissue kit (Qiagen, Cat: 69506). An additional centrifugation at 20,000 *g* for 1 min was included after the AW2 buffer wash. QIAamp Mini Spin Columns were placed into a clean 2 ml collection tube following each spin. After adding 50 µl of Buffer AE, an incubation period of 5 min at room temperature was added to the protocol. After elution from the column, the DNA was further diluted with 50 µl of Buffer AE and stored at −20°C.

2.4. Quantitative real-time PCR

Quantitative real-time PCR (qPCR) was performed blinded to participant information, and each sample was run in duplicate for each primer. The mean of the duplicate samples was calculated for later analysis. Each reaction consisted of mitochondrial gene-specific primers (2.5 µl/rxn), TaqMan™ Fast Advanced Master Mix (7.5 µl/rxn), and DNA isolated from plasma or EVs (3 µl/rxn), which equaled a total of 13 µl. Primers were designed against four different regions of the mitochondrial genome as described previously (Byappanahalli et al., 2023, 2024; Lazo et al., 2021). Primer sequences are listed in Supplementary Table 1. qPCR was performed using a 7900HT Fast Real-Time PCR System (Applied Biosystems, software version SDS 2.4.1). The thermal profile consisted of the following: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 15 sec at 95 °C and 1 min at 60 °C. mtDNA levels were calculated as previously reported (Byappanahalli et al., 2023, 2024; Lazo et al., 2021). mtDNA values were $\log_2$ transformed as they were positively skewed.

2.5. Size Exclusion Chromatography EV Isolation

Size exclusion chromatography (SEC) was used to fractionate plasma using an Automatic Fraction Collector (AFC) (IZON, Cat: AFC-V2) fixed with an qEV Original 70 nm Gen2 column (IZON, Cat:ICO70). Before adding the plasma, the column was flushed with 15 ml of filtered 1X PBS. Plasma (0.4 ml) was added to the column and fractionated according to manufacturer's instructions. The settings were adjusted to collect: 2.5 ml buffer volume, 0.5 ml collection volume, and 10 fractions. Fractions 1–3 were combined and aliquoted for Nanoparticle Tracking Analysis (100 µl), subsequent DNA isolation (155 µl), whole EVs (300 µl) and stored at −80 °C until further use. The remainder of the EVs (945 µl) were concentrated as described below. The column was cleaned after each use with 8.5 ml of 0.5 M NaOH and were reused 5 times, according to the manufacturer's recommendations.

2.6. Concentrating of EVs

SEC-isolated EVs (945 µl total volume) were concentrated using an Amicon Ultra 10KDa-0.5 ml Centrifugal Filter Unit (Cat: UFC501008). Samples were loaded into the filter and centrifuged at 14,000 *g* for 15 mins. Steps were repeated to concentrate the total sample volume (945 µl). Collection of concentrated EV sample was achieved by inverting the filter and centrifuging for 2 min at 2000 *g* in a new tube. The concentrated EV sample was lysed in a 10X lysis buffer (10X tris-buffered saline (TBS), 10 % TritonX-100, 20 mM Ethylenediaminetetraacetic acid (EDTA) with protease and phosphatase inhibitors).

2.7. Immunoblotting

Lysates of concentrated EV samples and human umbilical vein endothelial cell (HUVEC) lysate were run on a 4–12 % NuPAGE Bis-Tris gel under sodium dodecyl sulfate (SDS)-denaturing conditions (Invitrogen ThermoFisher Scientific) and transferred onto polyvinylidene difluoride (PVDF). After blocking in 3 % BSA in TBS with 0.1 % Tween® 20 Detergent, the membrane was incubated with primary antibodies for 1 h at room temperature: CD9 (System Biosciences EXOAB-CD9A-1), Flotillin-1 (Abcam ab133497), and GM130 (Abcam ab52649). All primary antibodies were diluted 1:500. The membranes were incubated with the appropriate secondary horseradish peroxidase (HRP)-conjugated antibodies at 1:5000 dilution for 32 min. These blots were visualized using the KwikQuant Ultra HRP Substrate Solution (Kindle Biosciences, LLC; Cat: R1002) on an iBright 1500 system (ThermoFisher Scientific).

2.8. Nanoparticle Tracking Analysis

SEC-isolated EVs from above were diluted (1:5) into filtered 1x PBS. Some samples required further dilution, which was adjusted for in the final concentration. Nanoparticle Tracking Analysis (NTA) was performed using a NanoSight NS500 (Malvern Paralytical, software version NTA 3.4 Build 3.4.4) to analyze the size distribution and concentration of the EVs in each sample. Samples were recorded in five videos of 20 s at camera level 16 and threshold was adjusted between 3 and 4 to ensure adequate numbers of dots (6 or less) before beginning analysis. Samples were analyzed on the same machine by one user. EV concentration values were $\log_2$ transformed as the values were positively skewed.

2.9. Electron Microscopy

The Johns Hopkins University School of Medicine Microscope Facility conducted electron microscopy. SEC-isolated EVs were absorbed to freshly ionized 400 mesh formvar/carbon coated grids (Electron Microscopy Sciences, Cat: CF400-Cu-UL) and then washed with TBS (3 drops) and negatively stained in 1 % aqueous uranyl acetate. Images were then taken with a transmission electron microscope (ThermoFisher Talos L120C) at 120 kV using a ThermoFisher Ceta 16mP 16 bit CMOS camera.

2.10. Plasma EV DNA Isolation

A previously established protocol was followed for isolating plasma EV DNA (Byappanahalli et al., 2023; Lazo et al., 2021). In brief, SEC-isolated EVs (155 µl) were thawed and treated with 5 U DNase (Lucigen, Baseline-ZERO DNase, Cat: DB0715K) at 37 °C for 30 min. The DNase was inactivated with DNase Stop Solution during an incubation period of 10 min at 65 °C. EV DNA was isolated as described above for plasma DNA.

2.11. Multiplex proximity extension assay

Protein concentration of concentrated EV lysates (described above) was calculated using the Bradford Assay. Protein amounts were equalized so final concentration equaled 2.8 µg/µl in 40 µl. Proteins were analyzed by Proximity Extension Assay (PEA) technology using the Inflammation Panel by Olink® Proteomics (Olink® Proteomics). Experiments were performed blind. Two EV lysate samples were excluded due to low quality. Protein data are represented as normalized protein expression (NPX) units on a $\log_2$ scale, referred to here as normalized protein level (NPL). Out of the 92 proteins in the Olink® Inflammation panel, 29 proteins met our threshold for being detectable in more than 75 % of all the EV samples. These proteins are listed in Supplementary Table 2. Any missing protein values were excluded from the analysis. Values for Cystatin-D were positively skewed and thus $\log_2$ transformed

for analysis.

2.12. Statistical analysis

Statistical analysis was performed using R software version 4.4 (R Core Team, 2024). Pearson's chi-squared tests were used to analyze differences for sex, race, and poverty status across high and low perceived discrimination status. Student's *t*-test was used to analyze the differences between the groups for age. Correlations were assessed by Pearson correlation (pairwise complete observation). EV concentration, EV mtDNA, plasma mtDNA, and EV inflammatory protein levels were analyzed using linear regression, including variables for perceived discrimination, sex, race, poverty status, and age in decade units. Backward stepwise regression was used for all linear regression models starting with a full model considering all possible two-way interactions and statistical significance based on the relevant coefficient in the model. Non-significant interactions were eliminated until a final model, or base model of all main effects, was achieved. Presence of EV proteins were analyzed using threshold of detection data via logistic regression, including the study design of total perceived discrimination, sex, race, poverty status, and age in decade units. Statistical significance was defined as a p-value < 0.05 and adjustments for multiple comparisons were not performed due to the small sample size.

3. Results

3.1. Plasma mtDNA levels are associated with perceived discrimination, poverty, race, and age

Plasma DNA was isolated from participants who reported low or high perceived discrimination, balanced across sex and race (Table 1). Plasma mtDNA levels were analyzed by qPCR using four different mitochondrial specific primer sets that span the mitochondrial genome (Byappanahalli et al., 2023; Lazo et al., 2021). The four different primer sets are indicated in Supplementary Figure 1 and span unique regions of the mitochondrial genome:Mito_3164 spans the 16S rRNA (*MT-RNR2*) and tRNA-Ile1 (*MT-TL1*) genes, Mito_4625 the NADH dehydrogenase 2 (*MT-ND2*) gene region, Mito_7878 the Cytochrome c oxidase subunit 2

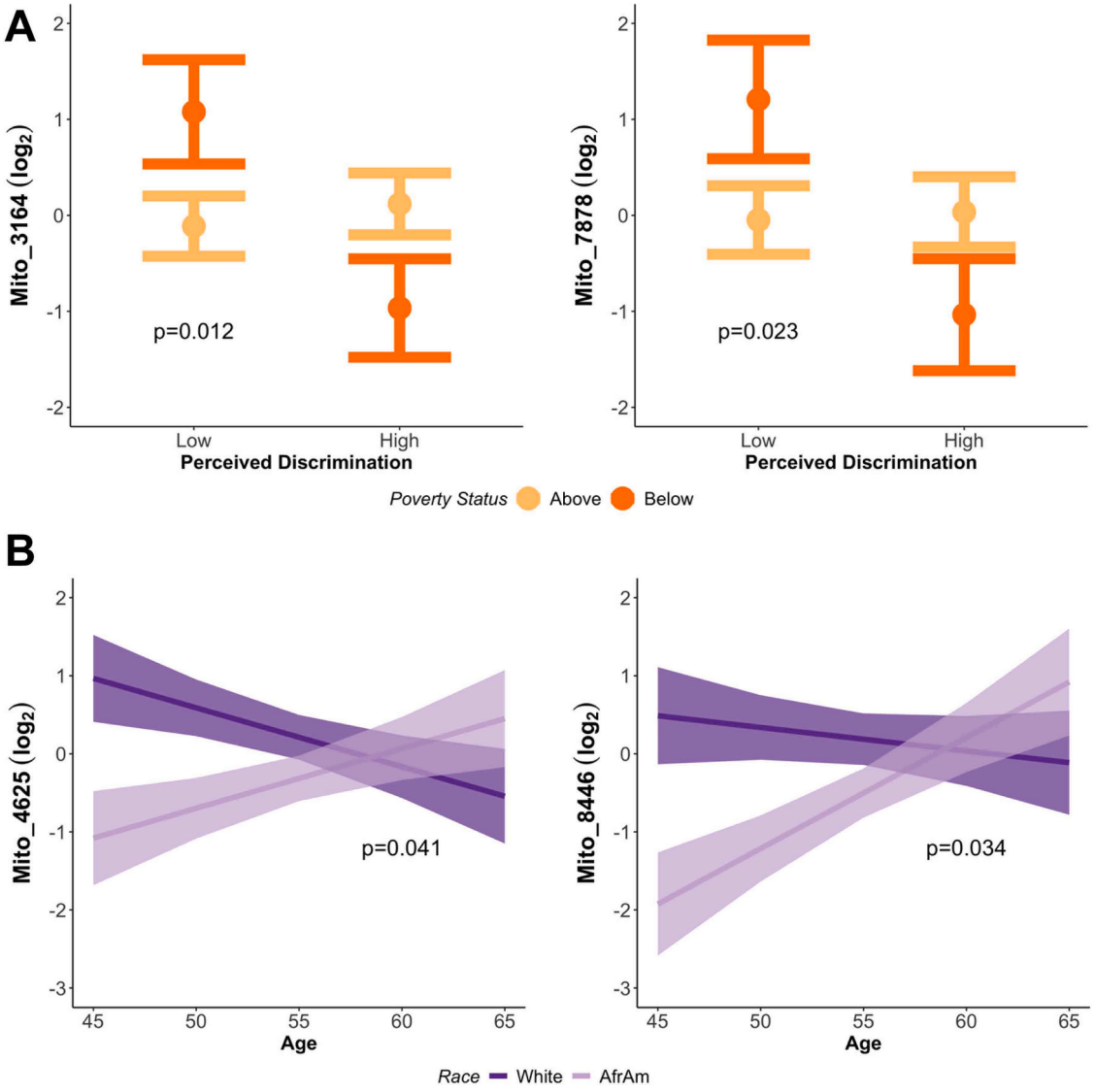

Fig. 1. Plasma mtDNA levels are higher in individuals experiencing low perceived discrimination and living below poverty. (A) DNA was isolated from plasma from cohort participants (n = 64) and mtDNA levels were quantified using four unique mtDNA-specific primers. Linear regression was used to examine the relationship between plasma mtDNA levels and perceived discrimination accounting for race, sex, poverty status, and age. Significant interactions between perceived discrimination and poverty status for Mito_3164 and Mito_7878 primer sets are shown. (B) Significant interactions between age and race were found for Mito_4625 and Mito_8446 primer sets are shown. The plots show the regression values ± standard error of estimated values.

(*COX2*) gene region, and Mito_8446 the ATP6/8 (MT-ATP8) gene region. Pearson correlation was used to examine the relationships between the different primer sets. All primer sets were significantly correlated with each other, confirming the presence of ccf-mtDNA and the relationship between the mtDNA regions in the circulation (Supplementary Fig. 2).

Linear regression was used to analyze the association between plasma mtDNA levels and perceived discrimination, accounting for race, sex, poverty status, and age. For two mitochondrial regions, Mito_3164 and Mito_7878, participants living below poverty who reported low perceived discrimination had higher levels of plasma mtDNA compared to those who reported high perceived discrimination (Fig. 1A). Plasma mtDNA levels were similar for those living above poverty regardless of discrimination level. For the other two mtDNA regions (Mito_4625 and Mito_8446) we found interactions between age and race (Fig. 1B). At lower ages, African American individuals had lower levels of plasma mtDNA than White individuals (Fig. 1B). Significant interactions of plasma mtDNA regions with demographic variables were also observed (Supplementary Table 3).

3.2. EV mtDNA levels are associated with perceived discrimination and poverty

Given that ccf-mtDNA can be encapsulated in EVs (Lazo et al., 2021), we next explored whether ccf-mtDNA in EVs was associated with perceived discrimination. EVs were isolated using size exclusion chromatography and analyzed according to Minimal Information for Studies of Extracellular Vesicles (MISEV) guidelines (Welsh et al., 2024). First, EV protein markers were validated by immunoblotting the EV-enriched fractions (combined F1–3) with the well-established EV markers, Flotillin-1 and CD9. Flotillin-1 and CD9 were present in EV fractions, while the purity marker GM130 was absent (Fig. 2A). Electron microscopy images of EV-enriched fractions showed round membrane-bound vesicles in the characteristic size range of EVs, along with cup-like vesicles which often occurs during the dehydration process for EM imaging (Fig. 2B). Next, EV size and concentration were examined using Nanoparticle Tracking Analysis (NTA). A typical size distribution of plasma EVs was observed (Fig. 2C). Linear regression was used to examine the relationship between EV concentration and perceived discrimination, race sex, poverty status, and age. We found that EV concentration was significantly higher in White participants compared to African American participants (Fig. 2D).

To analyze EV mtDNA levels, EVs were first DNase treated to remove DNA that may be on the outside of the EVs or that may coprecipitate during the separation process. Next, DNA was isolated and EV mtDNA levels were analyzed as described above for plasma mtDNA. EV mtDNA levels across regions were significantly correlated with each other (Supplementary Fig. 3) and plasma and EV mtDNA levels for the same mtDNA region were significantly correlated (Supplementary Fig. 4), indicating that ccf-mtDNA is present and detectable in both plasma and EVs from the participants in this cohort.

Linear regression was used to assess the four mtDNA regions in

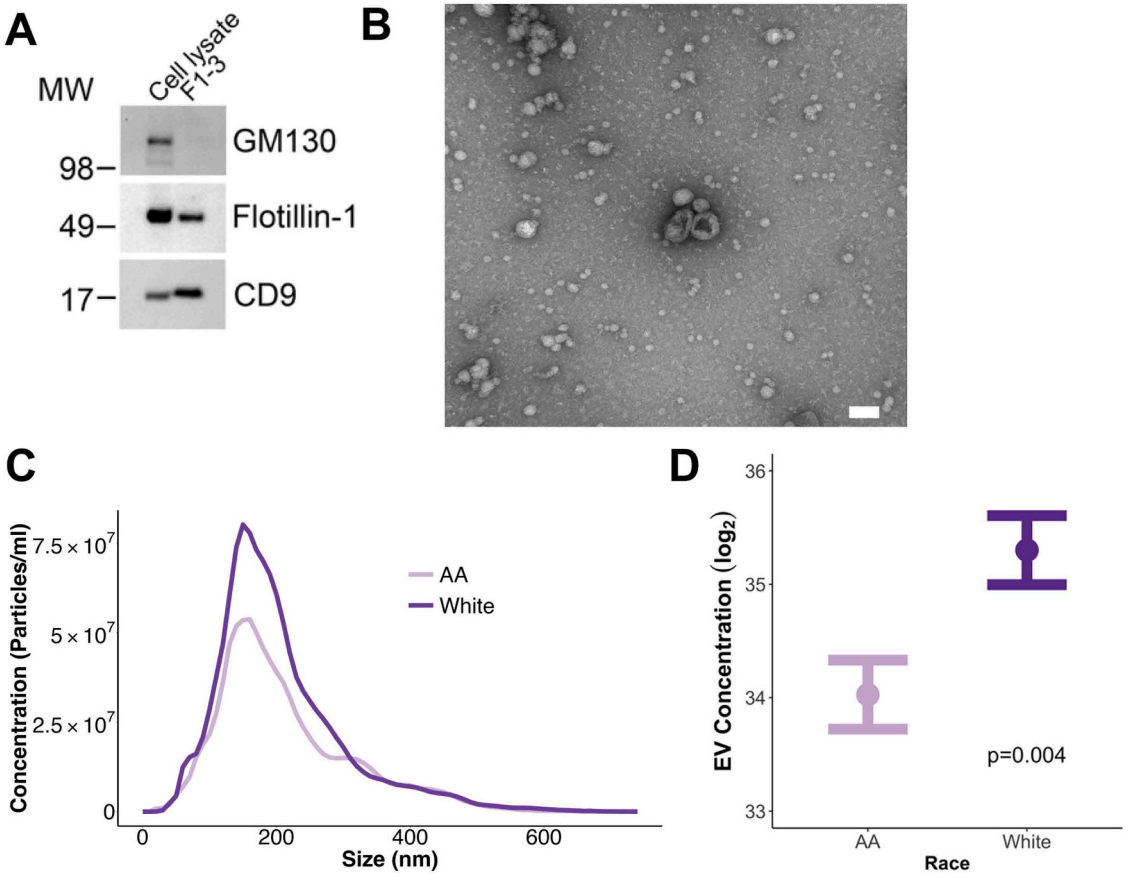

Fig. 2. EV characteristics of perceived discrimination cohort. Plasma EVs were isolated from participants who reported high or low perceived discrimination (n = 64) using size exclusion chromatography. Fractions 1–3 (F1–3) were pooled for the (EV)-enriched fractions. A) Plasma EVs (F1–3) and human umbilical vein endothelial cells were lysed and immunoblotted with antibodies against GM130, Flotillin-1, and CD9. GM130 served as a negative control, while Flotillin-1 and CD9 are known EV markers. C) EV morphology and size were visualized using electron microscopy (scale bar = 100 nm). C) EV size and distribution were analyzed using nanoparticle tracking analysis. The distribution was averaged for each group (AA=African American). D) EV concentration values were log2 transformed. Linear regression was used to examine the relationship between EV concentration and perceived discrimination accounting for race, sex, poverty status, and age (n = 64). The plot shows the regression values ± standard error of estimated values.

relation to perceived discrimination, race, sex, poverty status, and age (Fig. 3). For three mtDNA regions (Mito_3164, Mito_7878, and Mito_8446), participants who lived below poverty status had higher levels than those living above poverty status among participants who reported low perceived discrimination. Only for the mtDNA region Mito_7878 was this difference between poverty levels also significant for those who reported high discrimination. Similar to the results for plasma mtDNA, EV mtDNA levels were higher for adults who reported low perceived discrimination compared to those who reported high discrimination for people living below poverty status for Mito_7878. For Mito_3164 and Mito_7878, the findings were in the same direction, but not significant in post hoc testing (p-values of 0.100 and 0.054 respectfully). There was also a significant main effect of age for EV Mito_8446 (Supplementary Table 4). Overall, these results indicate that EV mtDNA levels are associated with perceived discrimination and poverty.

3.3. EV inflammatory proteins are associated with perceived discrimination, age, race, and sex

Given that psychosocial stress, including perceived discrimination, is associated with inflammation (Lewis et al., 2010) and EVs can carry inflammatory protein cargo (Byappanahalli et al., 2023; Noren Hooten et al., 2019; Wu et al., 2020), we quantified inflammatory proteins in EVs using PEA technology. The initial analyses only included the 29 inflammatory proteins detectable in more than 75 % of all the EV samples. These proteins are listed in Supplementary Table 2. Regression analyses revealed significant findings for IL-18 and Cystatin-D. There was a significant interaction for EV IL-18 levels between perceived discrimination and age. For participants over age 55, EV IL-18 levels were higher for those who reported high perceived discrimination compared to those who reported low discrimination (Fig. 4A). EV Cystatin-D levels revealed a significant interaction between perceived discrimination and race (Fig. 4B). EV Cystatin-D levels were higher in White individuals reporting high perceived discrimination compared to African American individuals reporting high perceived discrimination

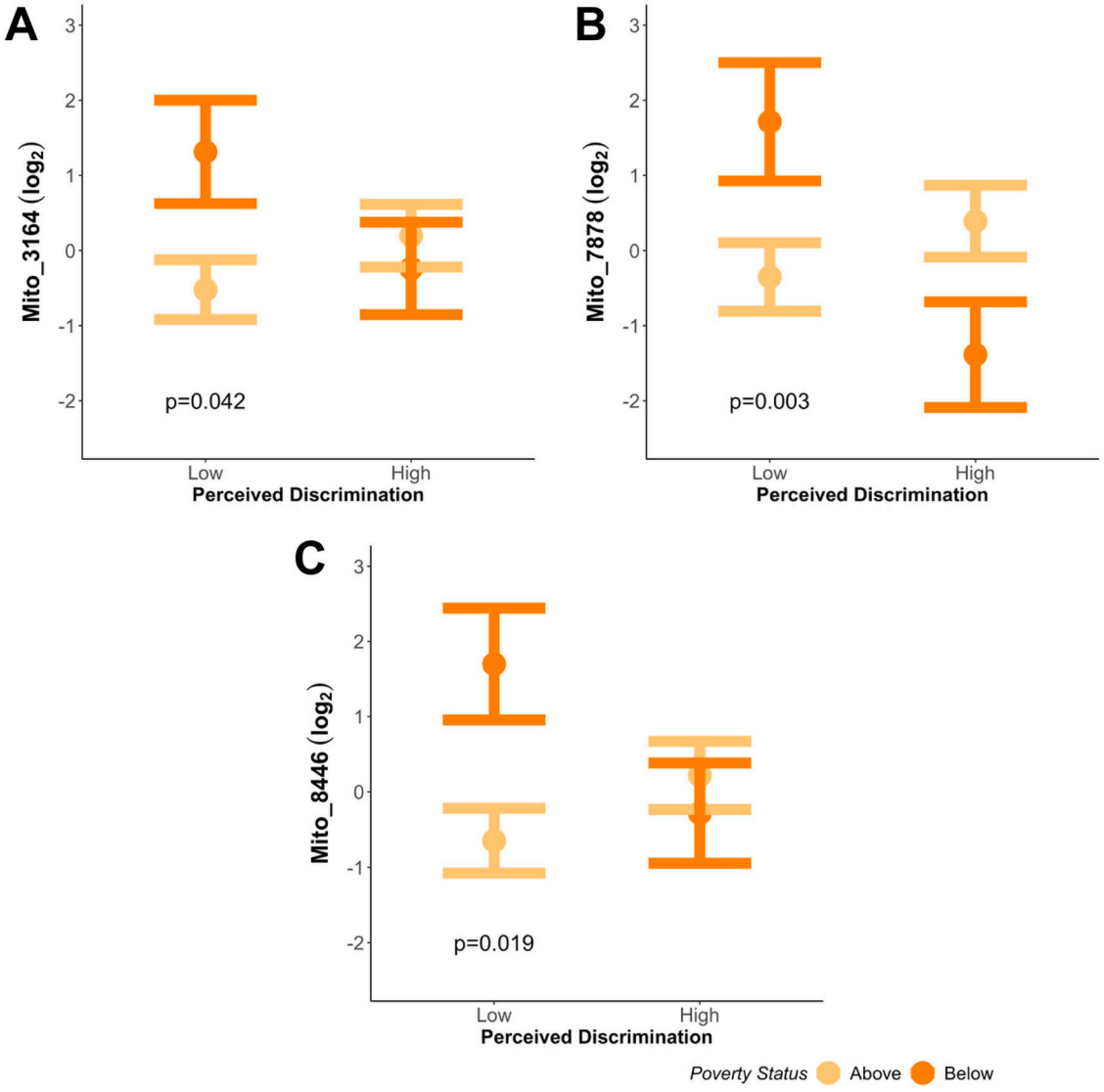

Fig. 3. EV mtDNA levels are associated with perceived discrimination and poverty status. Plasma EVs were isolated from cohort participants (n = 64) and subsequently DNA was isolated from EVs. EV mtDNA levels were quantified using four unique mtDNA-specific primers. Linear regression was used to examine the relationship between EV mtDNA levels and perceived discrimination accounting for race, sex, poverty status, and age. Significant interactions between perceived discrimination and poverty status for the **A)** Mito_3164 **B)** Mito_7878 and **C)** Mito_8446 primer sets are shown. The plots show the regression values ± standard error of estimated values.

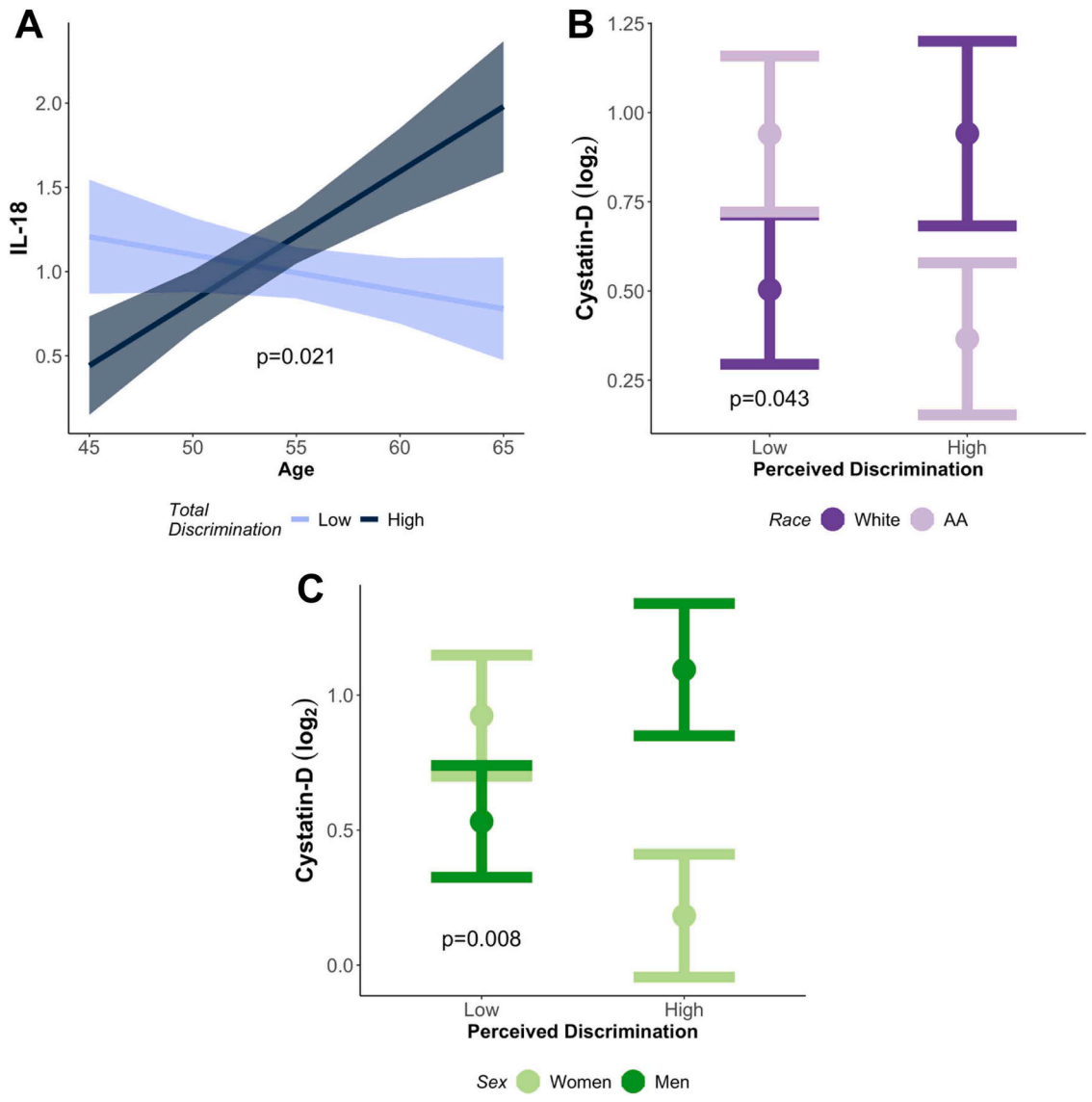

Fig. 4. EV inflammatory proteins are associated with perceived discrimination and age, race, and sex. Plasma EVs from the discrimination cohort were lysed and analyzed in a multiplex proximity extension assay (n = 62). Linear regression was used to examine the relationship between plasma mtDNA levels and perceived discrimination accounting for race, sex, poverty status, and age. A) EV IL-18 had a significant interaction with perceived discrimination and age. EV Cystatin-D had a significant interaction with B) perceived discrimination and race C) and perceived discrimination and sex. The plots show the regression values ± standard error of estimated values.

(Fig. 4B). In addition, for EV Cystatin-D levels there was a significant interaction between perceived discrimination and sex (Fig. 4C). Men who reported high discrimination had higher levels of EV Cystatin-D compared to women who reported high discrimination (Fig. 4C). Significant interactions of EV inflammatory proteins with demographic variables were also observed (Supplementary Table 5).

To examine all 92 proteins in the inflammatory panel with the variables of interest, each protein sample was converted to presence or absence. Logistic regression analyzed whether the presence of each EV inflammatory protein was associated with perceived discrimination, race, sex, poverty status, and age. The results revealed four inflammatory proteins which had interactions with perceived discrimination: Caspase-8 (CASP), Tumor necrosis factor (TNF), Interleukin-8 (IL-8), and TNF-related weak inducer of apoptosis (TWEAK) (also known as Tumor necrosis factor receptor superfamily member 12 A (TNFRSF12A) (Fig. 5). The presence of the first two proteins, Caspase-8 and TNF, were significantly related to an interaction of poverty status and perceived discrimination although in different directions. Among the participants who reported high perceived discrimination, those living below poverty status were more likely to have detectable levels of EV Caspase-8 than those living above poverty status. While for TNF, participants who reported high perceived discrimination living below poverty status were less likely to have detectable levels of EV TNF than those living above poverty status. The proteins IL-8 and TWEAK were significantly related to interactions between sex and perceived discrimination. Among participants who reported low perceived discrimination, women were more likely to have detectable levels of EV IL-8 than men. For TWEAK, we observed the opposite relationship. Among those reporting low perceived discrimination, men were more likely to have detectable levels of EV TWEAK than women. Logistic regression analysis found that the presence of EV inflammatory proteins was also associated with demographic variables (Supplementary Table 6).

We further analyzed the relationship between plasma and EV mtDNA levels with EV inflammatory proteins using pairwise Pearson correlation for the 29 proteins, which met the detectable criteria (Supplementary Fig. 5). Plasma and EV mtDNA levels were significantly correlated.

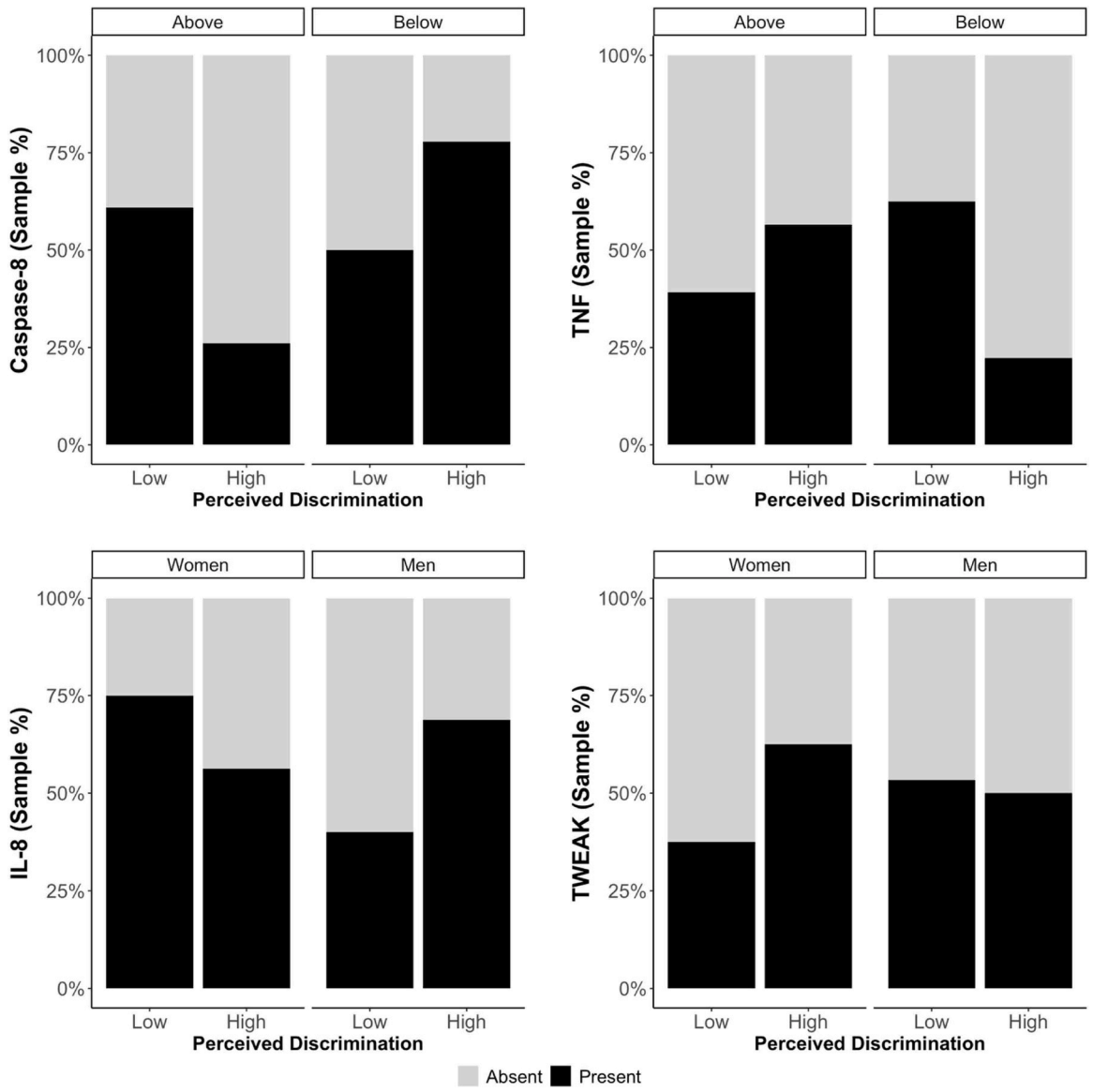

Fig. 5. The presence of EV inflammatory proteins are associated with perceived discrimination, sex, and poverty status. Plasma EVs from the discrimination cohort were lysed and analyzed using a multiplex proximity extension assay (n = 62). Logistic regression was used to assess the presence of each inflammatory protein in EVs with perceived discrimination, race, sex, poverty status, and age.

Plasma and EV mtDNA levels were positively, significantly correlated with several EV inflammatory proteins including: CD244, CD40, CXCL1, CXCL11, CXCL5, CXCL6, IL-18, MCP-1, MMP-1, ST1A1, and STAMBP. EV VEGF was only correlated with EV Mito_3164 and EV Mito_4625. Plasma and EV mtDNA levels were negatively correlated with CSF-1, DNER, TRAIL, and OPG. Interestingly, EV mtDNA, but not plasma mtDNA, was negatively correlated with EV IL-1α levels. Also, other significant associations were found for specific mtDNA regions and EV inflammatory proteins as visualized in Supplementary Figure 5.

We also wanted to examine the relationship between plasma and EV mtDNA levels and clinical inflammatory biomarkers. Using Pearson correlation, we found that neither plasma nor EV mtDNA were associated with any of the clinical inflammatory biomarkers assessed (Supplementary Fig. 6). Therefore, plasma and EV mtDNA may be indicators of different molecular pathways than those related to the clinical inflammatory biomarkers.

4. Discussion

In this study, we examined the association of plasma ccf-mtDNA and EV mtDNA and inflammatory proteins with perceived discrimination in a middle-aged cohort of African American and White adults. Using an intersectional framework, we report that among individuals living below poverty, those with higher perceived discrimination had lower ccf-mtDNA levels in both plasma and EVs than those with low perceived discrimination. At younger ages, ccf- mtDNA levels were lower in African American participants than White participants. These levels were higher at advanced age, but only in African American participants. We also examined inflammatory proteins in EVs and found that EV IL-18 and Cystatin-D were associated with perceived discrimination and age, sex, or race. The presence of EV Caspase-8 and TNF were associated with perceived discrimination and poverty, while EV IL-8 and TWEAK were associated with perceived discrimination and sex.

Emerging evidence indicates that mitochondria are stress mediators that sense, integrate, and respond to stress (Picard and McEwen, 2018a). It is thought that mitochondria release ccf-mtDNA in response to cellular

damage or stress as part of the mitochondrial-derived DAMP response. However, we do not fully understand the conditions that result in mtDNA release. Furthermore, ccf-mtDNA can also be detected in the circulation in whole mitochondria (Al Amir Dache et al., 2020; Song et al., 2020; Stephens et al., 2020) or in EVs (Lazo et al., 2021; Sansone et al., 2017), which may be the consequence of different mechanisms that package mtDNA into EVs and extrude them into the extracellular environment (Trumpff et al., 2021). Other forms of ccf-mtDNA in plasma also have been described and would be interesting to examine in future studies (Brestoff et al., 2025; Caicedo et al., 2024). Recent data have shown that in humans, serum/plasma ccf-mtDNA was elevated after experiencing a laboratory-setting psychological challenge to induce acute stress (Hummel et al., 2018; Trumpff et al., 2019). Cell-based experiments indicated that one mechanism that may result in ccf-mtDNA may be glucocorticoid signaling (Trumpff et al., 2019). Acute stress induced by exercise also alters ccf-mtDNA levels, but these results are not consistent and vary due to types of exercise, timing of blood sampling, small sample size, and differences in the blood processing (reviewed in (Trumpff et al., 2021)). Therefore, we are only beginning to understand the different forms of ccf-mtDNA in plasma and the mechanisms of release of these molecules.

Our data indicate an interaction of poverty and discrimination with ccf-mtDNA levels in plasma and EVs. In general, ccf-mtDNA in plasma and EVs was lowest among those living below poverty and experiencing high discrimination, and highest among those living below poverty experiencing low discrimination. Perhaps chronic stress from poverty and discrimination, like other negative psychosocial factors, result in lower cellular mitochondrial abundance or function, which may lead to lower circulating levels of mtDNA. Our perceived discrimination data are derived from questionaries focused on lifetime and racial discrimination and gather information about chronic long-term perceived discrimination. Poverty was denoted at the time of enrollment into HANDLS. Therefore, it can only be considered as a current measure of poverty, not necessarily a lifetime exposure. It is feasible to also conjecture that individuals exposed to a lifetime of high perceived discrimination may acquire an adaptive response to stress, and the added stress of poverty does not further physiologically affect ccf-mtDNA levels. This may explain why those living both above and below poverty with high levels of perceived discrimination have similar levels of ccf-mtDNA. Given the observational nature of this study, we cannot assess the directionality of the findings as to whether psychosocial stress affects mitochondrial biology or if mitochondrial function affects the perception of stress and reporting of it. Nevertheless, these findings are important given the emerging interest in biological mechanisms that may drive how psychosocial factors affect health and well-being.

We report differences in EV inflammatory proteins with discrimination and age, race, or sex. Higher levels of inflammation have also been associated with discrimination (reviewed in (Agbonlahor et al., 2024)). However, in these studies, inflammation was generally assessed by examining levels of either high-sensitivity C-reactive protein or IL-6. Thus, other inflammatory markers have not been analyzed in the context of discrimination, especially those in EVs. Chronic inflammation is an established driver of age-related disease. EV IL-18 levels were higher with high discrimination, but only in those adults over the age of 55. IL-18 is a pro-inflammatory cytokine that plays a role in many inflammatory and autoimmune diseases. IL-18 is also part of the host defense system and the innate immune response (Landy et al., 2024). Interestingly, endogenous stressors utilize DAMPS, such as ccf-mtDNA, to initiate an innate immune response via activation of the inflammasome, which in turn activates IL-18 (Landy et al., 2024). In our data, EV IL-18 levels are higher with advancing age, but only in those with high perceived discrimination. Therefore, the lifetime effects of perceived discrimination may persist in activating “sterile inflammation” and contribute to the chronic inflammation, termed “inflammaging,” observed in older individuals. Here, high perceived discrimination would further enhance these pathways to promote chronic, sterile inflammation, that may lead to susceptibility to age-related chronic disease or infection.

EV Cystatin-D levels were higher in White individuals reporting high perceived discrimination compared to African American individuals reporting high perceived discrimination. In addition, men who reported high discrimination had higher levels of EV Cystatin-D compared to women who reported high discrimination. Cystatin-D is a member of the Type 2 cystatins, which are cysteine protease inhibitors (Zhang and Zhan, 2023). It acts as a tumor suppressor that also modulates gene and protein expression and is involved in tumorigenesis and neurodegeneration (Alvarez-Diaz et al., 2009; Ferrer-Mayorga et al., 2015). Serum levels were recently associated with traumatic brain injury (Hill et al., 2017), although less is understood about this Type 2 cystatin, and therefore, we can only speculate on its role in discrimination. We also observed interactions of other inflammatory proteins including EV Caspase-8 and TNF with perceived discrimination and poverty. Among the participants who reported high perceived discrimination, those living below poverty status were more likely to have detectable levels of EV Caspase-8 than those living above poverty status. While for TNF, participants who reported high perceived discrimination living below poverty status were less likely to have detectable levels of EV TNF than those living above poverty status. EV IL-8 and TWEAK were associated with perceived discrimination and sex. Among participants who reported low perceived discrimination, women were more likely to have detectable levels of EV IL-8 than men. For TWEAK, we observed the opposite relationship. Among those reporting low perceived discrimination, men were more likely to have detectable levels of EV TWEAK than women. Caspase 8 is a cysteine-aspartic protease involved in apoptosis and modulation of pro-inflammatory cytokines and inflammasomes (Zhang et al., 2024). This protease is associated with immune system disorders, neurodegenerative diseases and cancer. TNF is a proinflammatory cytokine that regulates inflammatory responses, metabolism, cell viability and cytokine production. It is associated with cancer, cardiovascular disease, metabolic disease and well as neurologic disorders (for review (Aggarwal, Gupta and Kim, 2012)). IL-8 is a proinflammatory cytokine that activates neutrophils, promotes angiogenesis, and proliferation in endothelial and cancer cells (Waugh and Wilson, 2008). It is involved in atherosclerotic cardiovascular disease, cancer as well as in the hyperinflammatory acute respiratory distress syndrome in COVID-19 (Apostolakis et al., 2009). The cytokine TWEAK regulates cell survival, proliferation, angiogenesis, and apoptosis by inducing proinflammatory signaling and gene expression (Kumar et al., 2009). It has been associated with the cardiovascular remodeling of cardiovascular disease, cancer, neurodegeneration, stroke, and systemic lupus erythematosus (Mendez-Barbero et al., 2020). It is interesting to note that each of these inflammatory proteins plays a role in several chronic disorders known to have higher incidence and adverse outcomes in the United States population who report high levels of exposure to discrimination.

This study should be viewed in the context of limitations. This is an observational cross-sectional study; thus, directionality or causality cannot be ascertained. In this study we did not include other psychosocial factors, which may also affect EVs and their cargo. Yet, little is known in this field about the relationship between psychosocial factors and EVs. Self-reported discrimination may be subject to desirability bias. The sample size is in line with other EV-based studies (reviewed in (Noren Hooten et al., 2022a)), but may be limited in power to detect some differences. Our study has several notable strengths. Very little is known about how psychosocial factors, including discrimination, negatively affect health and well-being and few include both African American and White adults, especially in EV-based studies. Therefore, this study is novel in its approach to studying a potential biomarker of perceived discrimination and in its intersectional design to include a cohort of African American and White men and women living above and below poverty. Our findings suggest that discrimination interacts with

race, poverty, and sex to influence ccf-mtDNA levels in plasma and EVs and EV inflammatory proteins. Exploring possible biological pathways that underlie the adverse health outcomes associated with discrimination through an intersectional approach facilitates the inclusion of all relevant environmental and social identities and factors that might drive differential health outcomes possibly a valuable approach to use in other research that addresses population health differences (Holman et al., 2021). Therefore, these data provide a molecular mechanism whereby perceived discrimination may affect physiological systems that may negatively impact health.

Disclosures

The authors declare that they have no competing interests.

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CRediT authorship contribution statement

Nicole Noren Hooten: Writing – original draft, Methodology, Conceptualization. **Hannah E. Maul:** Writing – review & editing, Visualization, Formal analysis, Data curation. **Nicolle A. Mode:** Writing – review & editing, Visualization, Formal analysis, Data curation, Conceptualization. **Ngozi Ezike:** Writing – review & editing, Methodology, Data curation. **Tayla D. Hunter:** Writing – review & editing, Writing – original draft, Visualization, Formal analysis, Data curation. **Danielle L. Beatty Moody:** Writing – review & editing, Data curation, Conceptualization. **Alan B. Zonderman:** Writing – review & editing, Supervision, Formal analysis, Conceptualization. **Evans Michele Kim:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of Competing Interest

The authors have no conflicting interests to report.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.psyneuen.2025.107622.

Data availability

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request through the HANDLS website https://handls.nih.gov/.

References

Agbonlahor, O., et al., 2024. Racial/Ethnic discrimination and cardiometabolic diseases: a systematic review. J. Racial Ethn. Health Disparities 11, 783–807.
Aggarwal, B.B., Gupta, S.C., Kim, J.H., 2012. Historical perspectives on tumor necrosis factor and its superfamily: 25 years later, a golden journey. Blood 119, 651–665.
Al Amir Dache, Z., et al., 2020. Blood contains circulating cell-free respiratory competent mitochondria. FASEB J. 34, 3616–3630.
Alvarez-Diaz, S., et al., 2009. Cystatin d is a candidate tumor suppressor gene induced by vitamin d in human colon cancer cells. J. Clin. Invest 119, 2343–2358.
Apostolakis, S., et al., 2009. Interleukin 8 and cardiovascular disease. Cardiovasc Res 84, 353–360.
Argentieri, M.A., et al., 2025. Integrating the environmental and genetic architectures of aging and mortality. Nat. Med 31, 1016–1025.
Barnes, L.L., et al., 2012. Perceived discrimination and cognition in older African Americans. J. Int. Neuropsychol. Soc. 18, 856–865.
Beatty Moody, D.L., et al., 2018. Everyday discrimination and metabolic syndrome incidence in a Racially/Ethnically diverse sample: study of women's health across the nation. Psychosom. Med 80, 114–121.
Beatty Moody, D.L., et al., 2019. Lifetime discrimination burden, racial discrimination, and subclinical cerebrovascular disease among African Americans. Health Psychol. 38, 63–74.
Bowleg, L., 2012. The problem with the phrase women and minorities: intersectionality-an important theoretical framework for public health. Am. J. Public Health 102, 1267–1273.
Boyapati, R.K., et al., 2017. Advances in the understanding of mitochondrial DNA as a pathogenic factor in inflammatory diseases. F1000Research 6.
Brestoff, J.R., et al., 2025. Recommendations for mitochondria transfer and transplantation nomenclature and characterization. Nat. Metab. 7, 53–67.
Byappanahalli, A.M., et al., 2023. Mitochondrial DNA and inflammatory proteins are higher in extracellular vesicles from frail individuals. Immun. Ageing 20, 6.
Byappanahalli, A.M., et al., 2024. Extracellular vesicle mitochondrial DNA levels are associated with race and mitochondrial DNA haplogroup. iScience 27, 108724.
Caicedo, A., et al., 2024. Decoding the nature and complexity of extracellular mtDNA: types and implications for health and disease. Mitochondrion 75, 101848.
Dixson, A.C., et al., 2023. Context-specific regulation of extracellular vesicle biogenesis and cargo selection. Nat. Rev. Mol. Cell Biol. 24, 454–476.
Evans, M.K., et al., 2010. Healthy aging in neighborhoods of diversity across the life span (HANDLS): overcoming barriers to implementing a longitudinal, epidemiologic, urban study of health, race, and socioeconomic status. Ethn. Dis. 20, 267–275.
Ferrer-Mayorga, G., et al., 2015. Cystatin d locates in the nucleus at sites of active transcription and modulates gene and protein expression. J. Biol. Chem. 290, 26533–26548.
Freeman, D.W., et al., 2020. Association between GDF15, poverty and mortality in urban middle-aged African American and White adults. PLoS One 15, e0237059.
Goosby, B.J., Cheadle, J.E., Mitchell, C., 2018. Stress- related biosocial mechanisms of discrimination and African American health inequities. Annu. Rev. Sociol. 44 (44), 319–340.
Hill, L.J., et al., 2017. Cystatin d (CST5): an ultra-early inflammatory biomarker of traumatic brain injury. Sci. Rep. 7, 5002.
Holman, D., et al., 2021. Can intersectionality help with understanding and tackling health inequalities? Perspectives of professional stakeholders. Health Res Policy Syst. 19, 97.
Howcroft, T.K., et al., 2013. The role of inflammation in age-related disease. Aging (Albany NY) 5, 84–93.
Hummel, E.M., et al., 2018. Cell-free DNA release under psychosocial and physical stress conditions. Transl. Psychiatry 8, 236.
Kalluri, R., LeBleu, V.S., 2020. The biology, function, and biomedical applications of exosomes. Science 367.
Kong, L., et al., 2023. Extracellular vesicles in mental disorders: a State-of-art review. Int J. Biol. Sci. 19, 1094–1109.
Krieger, N., 1990. Racial and gender discrimination: risk factors for high blood pressure? Soc. Sci. Med 30, 1273–1281.
Kumar, M., et al., 2009. TNF-like weak inducer of apoptosis (TWEAK) activates proinflammatory signaling pathways and gene expression through the activation of TGF-beta-activated kinase 1. J. Immunol. 182, 2439–2448.
Landy, E., et al., 2024. Biological and clinical roles of IL-18 in inflammatory diseases. Nat. Rev. Rheumatol. 20, 33–47.
Lazo, S., et al., 2021. Mitochondrial DNA in extracellular vesicles declines with age. Aging Cell 20, e13283.
Lewis, T.T., et al., 2010. Self-reported experiences of everyday discrimination are associated with elevated C-reactive protein levels in older African-American adults. Brain Behav. Immun. 24, 438–443.
Lindqvist, D., et al., 2018. Circulating cell-free mitochondrial DNA, but not leukocyte mitochondrial DNA copy number, is elevated in major depressive disorder. Neuropsychopharmacology 43, 1557–1564.
Lucien, F., et al., 2023. MIBlood-EV: minimal information to enhance the quality and reproducibility of blood extracellular vesicle research. J. Extra Vesicles 12, e12385.
Mainous, A.G., et al., 2024. Inflammation and poverty as individual and combined predictors of 15-year mortality risk in middle aged and older adults in the US. Front. Med. 10, 2023.
Mendez-Barbero, N., et al., 2020. Tumor necrosis Factor-Like weak inducer of apoptosis (TWEAK)/Fibroblast growth Factor-Inducible 14 (Fn14) axis in cardiovascular diseases: progress and challenges. Cells 9.
van Niel, G., et al., 2022. Challenges and directions in studying cell-cell communication by extracellular vesicles. Nat. Rev. Mol. Cell Biol. 23, 369–382.
Noren Hooten, N., et al., 2019. Association of extracellular vesicle protein cargo with race and clinical markers of mortality. Sci. Rep. 9, 17582.
Noren Hooten, N., et al., 2022b. The accelerated aging phenotype: the role of race and social determinants of health on aging. Ageing Res Rev. 73, 101536.
Noren Hooten, N., et al., 2022a. Influences of age, race, and sex on extracellular vesicle characteristics. Theranostics 12, 4459–4476.
Noren Hooten, N., et al., 2023. Inflammatory proteins are associated with mortality in a middle-aged diverse cohort. Clin. Transl. Med 13, e1412.

Noren Hooten, N., Evans, M.K., 2020. Extracellular vesicles as signaling mediators in type 2 diabetes mellitus. Am. J. Physiol. Cell Physiol. 318, C1189–C1199.
Picard, M., McEwen, B.S., 2018b. Psychological stress and mitochondria: a systematic review. Psychosom. Med 80, 141–153.
Picard, M., McEwen, B.S., 2018a. Psychological stress and mitochondria: a conceptual framework. Psychosom. Med 80, 126–140.
R Core Team, 2024. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ⟨https://www.R-project.org/⟩.
Routen, A., et al., 2023. Intersectionality in health equity research. BMJ 383, 2953.
Sansone, P., et al., 2017. Packaging and transfer of mitochondrial DNA via exosomes regulate escape from dormancy in hormonal therapy-resistant breast cancer. Proc. Natl. Acad. Sci. USA 114, E9066–E9075.
Simkus, A., Holtz, K.D., Twombly, E.C., 2024. An intersectionality framework for identifying relevant covariates in health equity research. Front Public Health 12, 1286121.
Singh, T., Newman, A.B., 2011. Inflammatory markers in population studies of aging. Ageing Res Rev. 10, 319–329.
Song, X., et al., 2020. Existence of circulating mitochondria in human and animal peripheral blood. Int. J. Mol. Sci. 21, 2122.
Stephens, O.R., et al., 2020. Characterization and origins of cell-free mitochondria in healthy murine and human blood. Mitochondrion 54, 102–112.
Trumpff, C., et al., 2019. Acute psychological stress increases serum circulating cell-free mitochondrial DNA. Psychoneuroendocrinology 106, 268–276.
Trumpff, C., et al., 2021. Stress and circulating cell-free mitochondrial DNA: a systematic review of human studies, physiological considerations, and technical recommendations. Mitochondrion 59, 225–245.
US Department of Health and Human Services, 2004. Annual Update of the HHS Poverty Guidelines. Fed. Regist. 69 (30), 7336–7338.
Waugh, D.J., Wilson, C., 2008. The interleukin-8 pathway in cancer. Clin. Cancer Res 14, 6735–6741.
Welsh, J.A., et al., 2024. Minimal information for studies of extracellular vesicles (MISEV2023): from basic to advanced approaches. J. Extra Vesicles 13, e12404.
Whitaker, K.M., et al., 2017. Experiences of discrimination and incident type 2 diabetes mellitus: the Multi-Ethnic study of atherosclerosis (MESA). Am. J. Epidemiol. 186, 445–455.
Wild, C.P., 2005. Complementing the genome with an "exposome": the outstanding challenge of environmental exposure measurement in molecular epidemiology. Cancer Epidemiol. Biomark. Prev. 14, 1847–1850.
Williams, D.R., et al., 2019. Understanding how discrimination can affect health. Health Serv. Res 54 (2), 1374–1388.
Williams, D.R., Mohammed, S.A., 2009. Discrimination and racial disparities in health: evidence and needed research. J. Behav. Med. 32, 20–47.
Wu, S.F., et al., 2020. Extracellular vesicles in diabetes mellitus induce alterations in endothelial cell morphology and migration. J. Transl. Med 18, 230.
Yanez-Mo, M., et al., 2015. Biological properties of extracellular vesicles and their physiological functions. J. Extra Vesicles 4, 27066.
Zahodne, L.B., Sol, K., Kraal, Z., 2017. Psychosocial pathways to Racial/Ethnic inequalities in Late-Life memory trajectories. J. Gerontol. Ser. B 74, 409–418.
Zhang, W., et al., 2024. Caspase-8 in inflammatory diseases: a potential therapeutic target. Cell Mol. Biol. Lett. 29, 130.
Zhang, Z., Zhan, F., 2023. Type 2 cystatins and their roles in the regulation of human immune response and cancer progression. Cancers (Basel) 15.

INTERNATIONAL SOCIETY for EXTRACELLULAR VESICLES

MIBlood-EV

Standardized Reporting Tool for Blood EV Research (Human)

STUDY INFORMATION

1.0 **Manuscript title**	Plasma and extracellular vesicle mitochondrial DNA levels are associated with a psychosocial stressor and poverty		
1.1 **Corresponding author (Name and Email)**	Michele K. Evans; me42v@nih.gov		
1.2 **Institution name**	National Institute on Aging, National Institutes of Health		
1.3 **Time period of experiment (years)**	1	1.4 **Number of samples**	64
1.5 **Cargo of interest**	✓ Vesicles ✓ Protein ☐ RNA ☐ DNA ✓ Other: mtDNA		
1.6 **Biospecimen type**	✓ Plasma ☐ Serum	1.7 **Biospecimen state**	Frozen
1.8 **Source of frozen specimens**	Prepared by the lab	1.9 **Years of collection (range)**	2004-2008

BLOOD COLLECTION AND PROCESSING

2.0 **Patient fasting status**		2.1 **Fasting length (e.g. hours/days)**	
2.2 **Anatomical access site**	Antecubital fossa	2.3 **Needle diameter (e.g. gauge)**	20 or 22
2.4 **Blood volume collected (mL)**	6		
2.5 **Plasma anticoagulant**	✓ EDTA ☐ Citrate ☐ Heparin ☐ Other:		
2.6 **Serum tube type**		2.7 **Serum clotting time (minutes)**	
2.8 **Time between collection and first centrifugation (range in hours)**	1-3		
2.9 **Transport temperature**	Room temp (~22°C)	2.10 **Transport condition of tubes**	Unknown
2.11 **Centrifuge brand and model**	Beckman Coulter Allegra X-14 Centrifuge		
2.12 **Bucket rotor type**	Swing	2.13 **Number of centrifugation cycles**	One
2.14 **1st Centrifugation speed (RCF in x g)**	1460	2.15 **1st Rotor brake**	Yes
2.16 **1st Centrifugation temperature**	Room temp (~22°C)	2.17 **2nd Centrifugation speed (RCF in x g)**	
2.18 **2nd Rotor brake**		2.19 **2nd Centrifugation temperature**	
2.20 **Additional processing steps (e.g. filtration)**			
2.21 **Storage tubes (brand, type, source, catalog number)**	Nunc 1.8 ml CryoTube Vials		
2.22 **Storage temperature**	-80°C	2.23 **Length of storage (range in years)**	15-19

PLASMA/SERUM QUALITY CONTROL

3.0 **Number of freeze-thaw cycles (range)**	1		
3.1 **Thawing temperature**	on ice	3.2 **Thawing duration (minutes)**	~60

Hemolysis

3.3 **Presence of hemolysis**	No	3.4 **Number of samples affected (e.g. <25%, 25-50%)**	0
3.5 **Method used**	Visual inspection	3.6 **RBC count (Median, 95% CI, N)**	
3.7 **RBC counter brand and type**			
3.8 **Spectrophotometry hemoglobin concentration (g/L)**			
3.9 **Spectrophotometer brand, model and wavelength measured (e.g. 414 nm)**			
3.10 **Hemolized samples were discarded**			

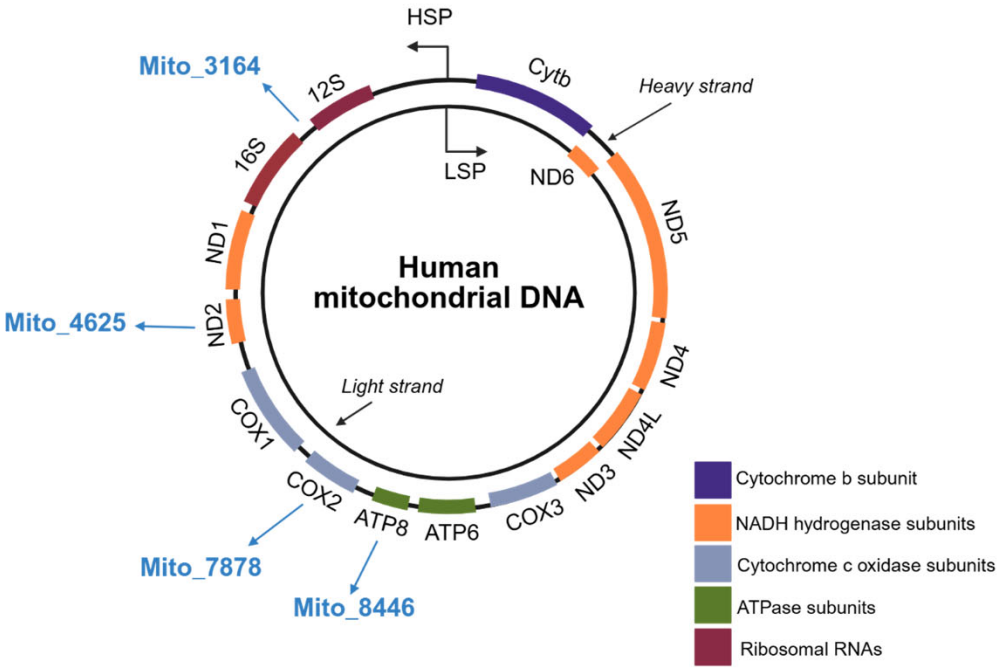
Platelets

3.11 **Presence of platelets**	Not Tested	3.12 **Method used (e.g. Flow Cytometry)**	
3.13 **Marker(s) used (e.g. CD61, CD41)**			
3.14 **Concentration (median, 95% CI, N)**			
3.15 **Platelet counter instrument brand and type**			
3.16 **Flow cytometer brand and type**			
3.17 **Flow cytometry size and fluorescence ranges of detection in nanometers and MESF, respectively**			

Lipoproteins

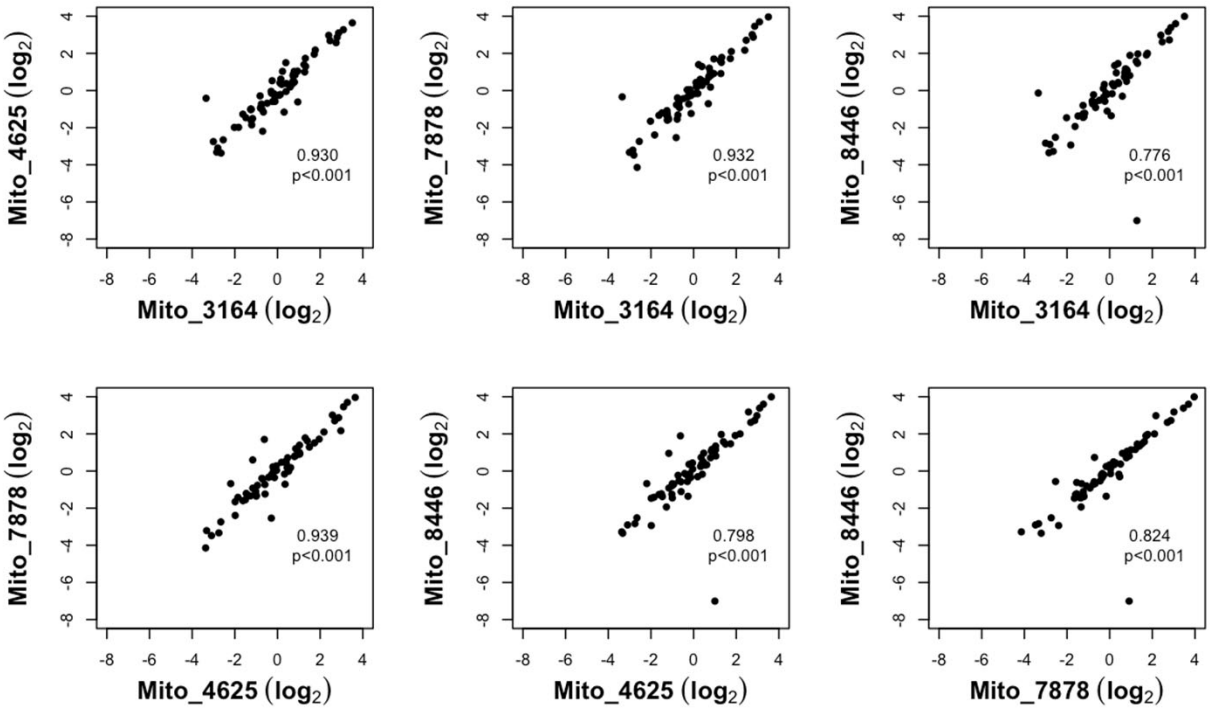
3.18 **Presence of lipoproteins**	Not tested	3.19 **Method used (WB, ELISA, FC)**	
3.20 **Spectrophotometry L-index**			
3.21 **Spectrophotometer brand, model and wavelength measured (e.g. 700 nm)**			
3.22 **WB Marker(s) used (e.g. Apo B)**			
3.23 **Western blot images provided in manuscript?**			
3.24 **Flow cytometry marker(s) used (e.g. ApoB)**			
3.25 **Flow cytometry concentration (median, 95% CI, N)**			
3.26 **Flow cytometer brand and type**			
3.27 **Flow cytometry size and fluorescence ranges of detection in nanometers and MESF, respectively**			

Supplementary Figure 1



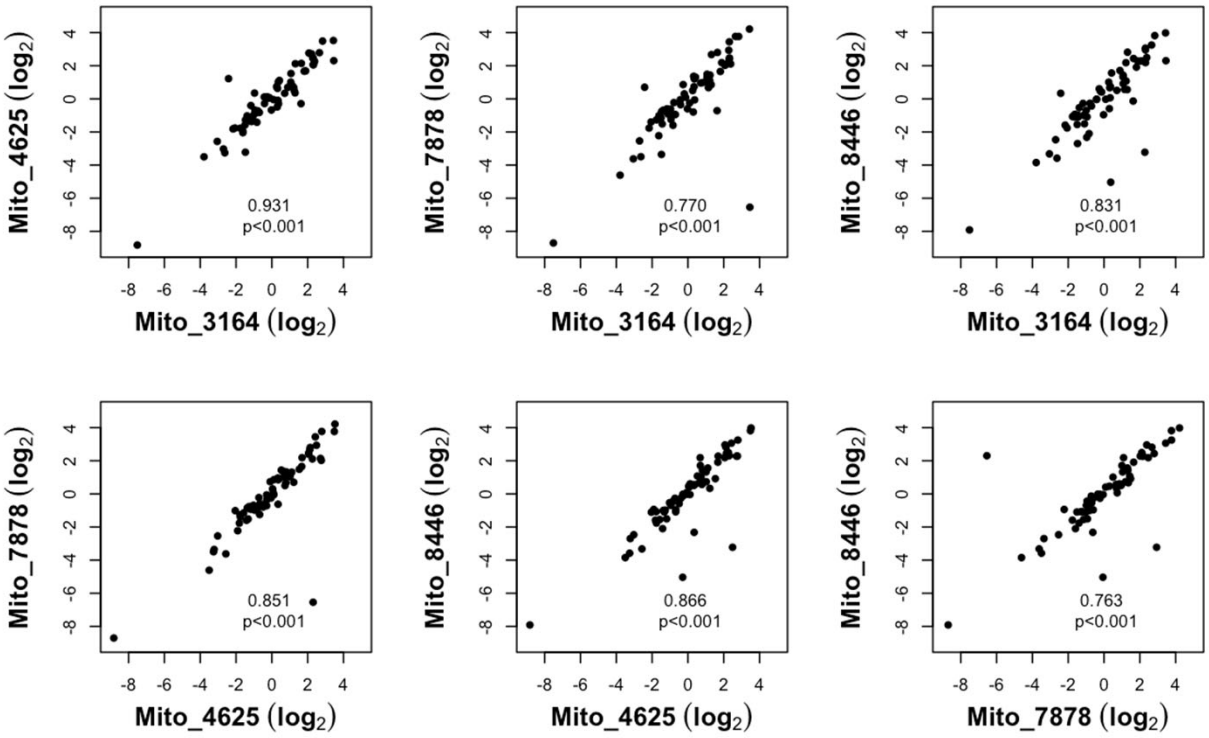
Supplementary Figure 1. Schematic of mitochondrial DNA and primers. Human mitochondrial genome with mtDNA primers indicated with blue arrow and primer name denoted by starting nucleotide. Mito_3164 spans the 16S rRNA (*MT-RNR2*) and tRNA-Ile1 (*MT-TL1*) genes, Mito_4625 the NADH dehydrogenase 2 (*MT-ND2*) gene region, Mito_7878 the Cytochrome c oxidase subunit 2 (*COX2*) gene region, and Mito_8446 the ATP6/8 (*MT-ATP8*) gene region. Created in BioRender. Noren Hooten, N. (2025) https://BioRender.com/tgh46ch

Supplementary Figure 2



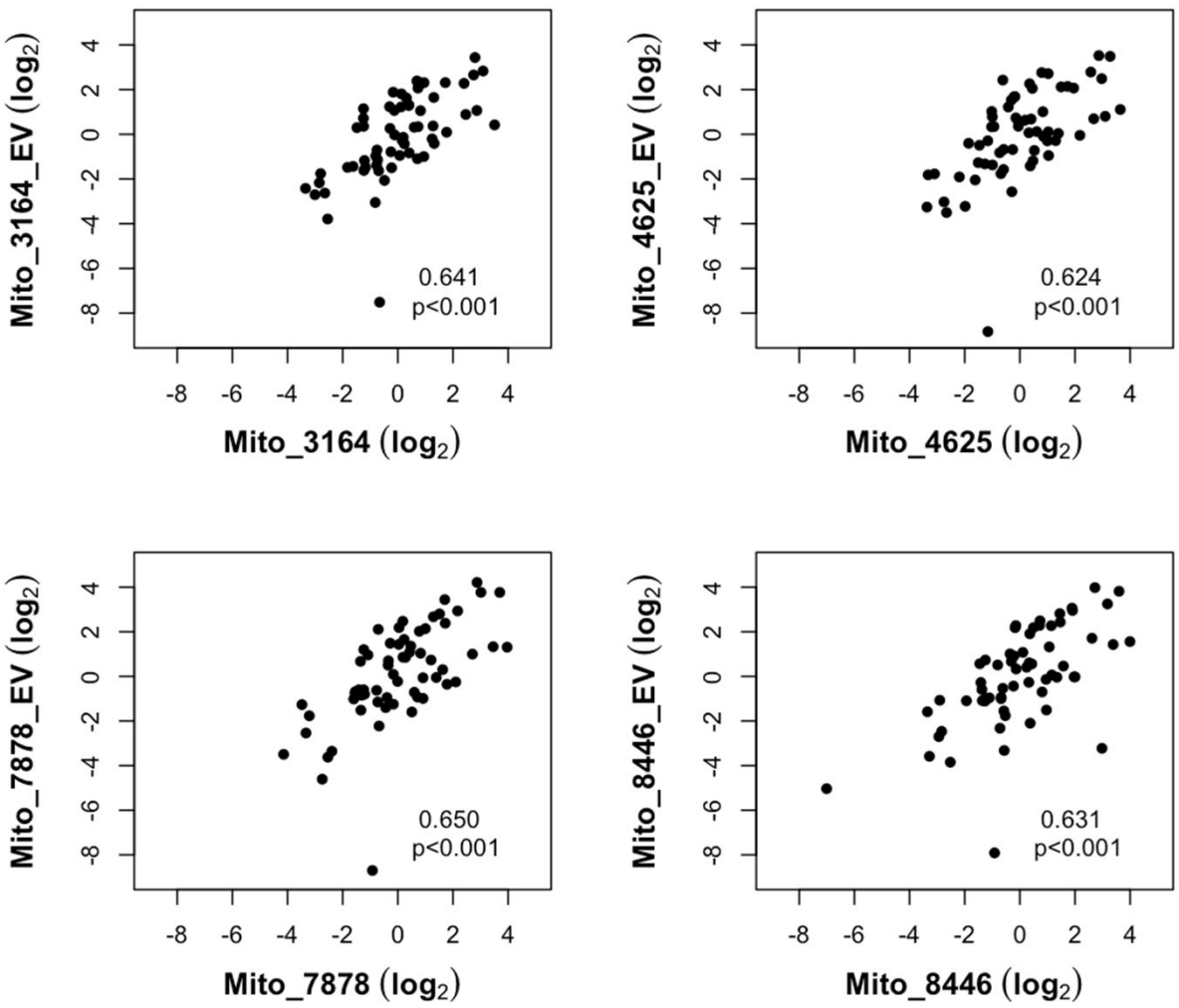
Supplementary Figure 2. Plasma mtDNA levels are significantly correlated. The relationship between plasma mtDNA levels (log_2 transformed) amplified with the four primer sets (Mito_3164, Mito_4625, Mito_7878, and Mito_8446) were analyzed using Pearson correlation. Pearson correlation (r) and p-values are displayed.

Supplementary Figure 3



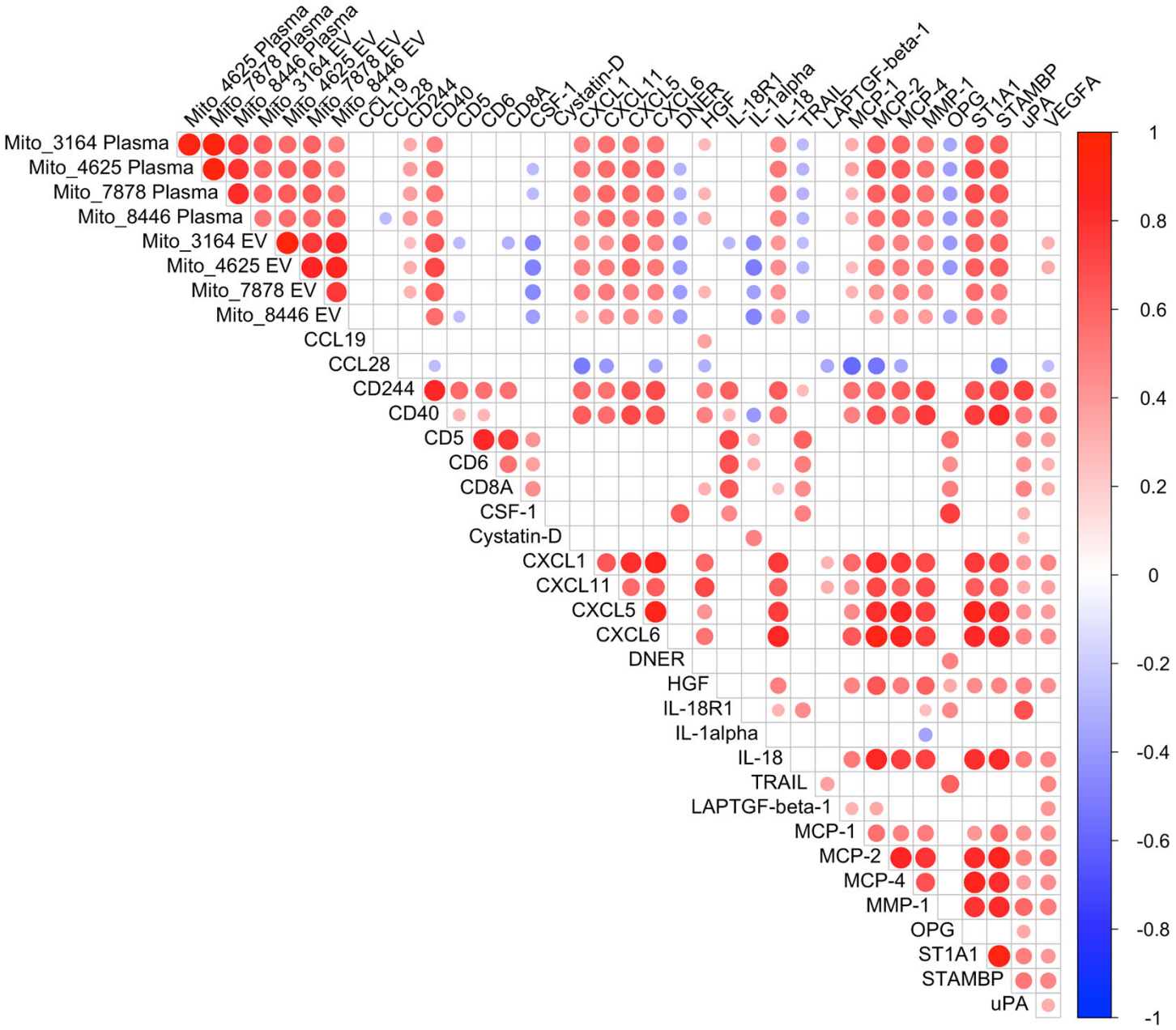
Supplementary Figure 3. EV mtDNA levels are significantly correlated. Plasma EVs were isolated from the discrimination cohort and then DNA was isolated from the EVs. EV mtDNA levels were amplified using four primer sets (Mito_3164, Mito_4625, Mito_7878, and Mito_8446). The relationship between EV mtDNA levels (log_2 transformed) amplified with the four primer sets were analyzed using Pearson correlation. Pearson correlation (r) and p-values are displayed.

Supplementary Figure 4



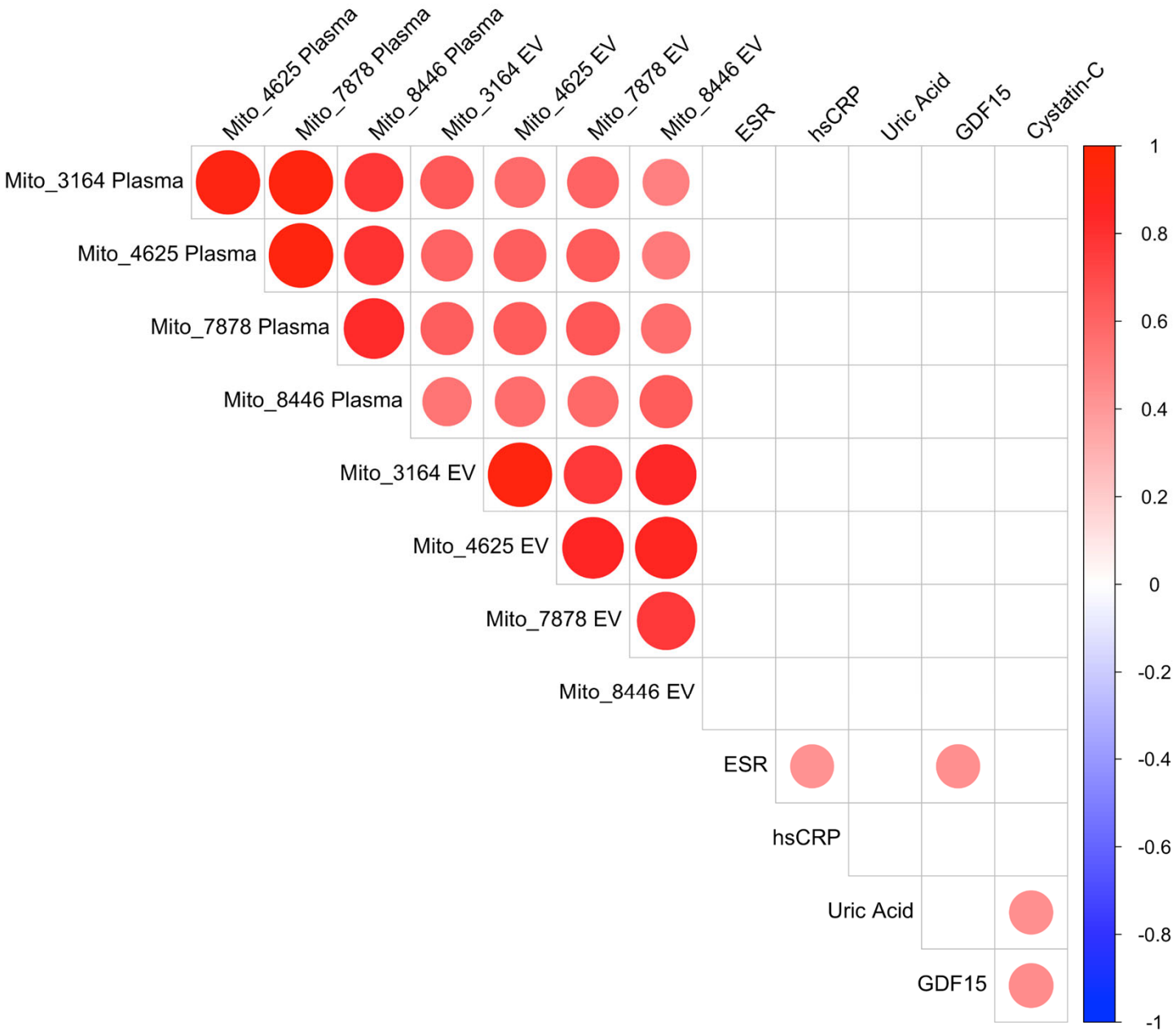
Supplementary Figure 4. Plasma and EV mtDNA levels are significantly correlated. mtDNA was amplified using four primer sets (Mito_3164, Mito_4625, Mito_7878, and Mito_8446) from plasma and from EVs from participants in the discrimination cohort. The relationship between plasma and EV mtDNA levels (log_2 transformed) were analyzed using Pearson correlation. Pearson correlation (r) and p-values are displayed.

Supplementary Figure 5



Supplementary Figure 5. Plasma and EV mtDNA levels are correlated with EV inflammatory proteins. Pearson correlation was used to determine the associations between plasma and EV mtDNA amplicon regions and EV inflammatory proteins. The four mitochondrial primer sets are indicated by "Mito_X". Circles indicate significance of $p<0.05$. The strength of the colors and the size of the circles correspond to the degree of significance. Color is based on correlation coefficient (r), where r = 1 is red and r = − 1 is blue.

Supplementary Figure 6



Supplementary Figure 6. Clinical inflammatory biomarkers do not correlate with plasma or EV mtDNA levels. Pearson correlation was used to determine the associations between plasma and EV mtDNA amplicon regions and clinical inflammatory biomarkers. The four mitochondrial primer sets are indicated by "Mito_X". Circles indicate significance of $p<0.05$. The strength of the colors and the size of the circles correspond to the degree of significance. Color is based on correlation coefficient (r), where r = 1 is red and r = − 1 is blue. hsCRP= High sensitivity C-reactive protein; ESR=erythrocyte sedimentation rate; GDF15=Growth differentiation factor 15

Supplementary Table 1. Primer sequences for qPCR

Gene	Primer Name	Forward and Reverse Sequence	Probe	Ref.
MT-RNR2/MT-TL1	Mito_3164	5'CCTTCCCCCGTAAATGATATCA3' / 5'GCCATCTTAACAAACCCTGTTCTT3'	5'FAM-AACTTAGTATTATACCCACACCC-MGB3'	1
MT-ND2	Mito_4625	5'CACAGAAGCTGCCATCAAGTA3' / 5'CCGGAGAGTATATTGTTGAAGAG3'	5'FAM-CCTCACGCAAGCAACCGCATCC-MGB3'	2
MT-COX2	Mito_7878	5'AATCAATTGGCGACCAATGG3' / 5'CGCCTGGTTCTAGGAATAATGG3'	5'FAM-ACTGAACCTACGAGTACAC-MGB3'	3
MT-ATP8	Mito_8446	5'AATATTAAACACAAACTACCACCTACCT3' / 5'TGGTTCTCAGGGTTTGTTATAA3'	5'FAM-CCTCACCAAAGCCCATA-MGB3'	4

References for Primer Sequences

1. Lazo S, Noren Hooten N, Green J, Eitan E, Mode NA, Liu Q-R, et al. Mitochondrial DNA in extracellular vesicles declines with age. Aging cell. 2021;20(1):e13283.

2. Pinti M, Cevenini E, Nasi M, De Biasi S, Salvioli S, Monti D, et al. Circulating mitochondrial DNA increases with age and is a familiar trait: Implications for "inflamm-aging". Eur J Immunol. 2014;44(5):1552-62.

3. Shao JY, Gao HY, Li YH, Zhang Y, Lu YY, Zeng YX. Quantitative detection of common deletion of mitochondrial DNA in hepatocellular carcinoma and hepatocellular nodular hyperplasia. World J Gastroenterol. 2004;10(11):1560-4.

4. Walker JA, Hedges DJ, Perodeau BP, Landry KE, Stoilova N, Laborde ME, et al. Multiplex polymerase chain reaction for simultaneous quantitation of human nuclear, mitochondrial, and male Y-chromosome DNA: application in human identification. Analytical Biochemistry. 2005;337(1):89-97.

Supplementary Table 2. Inflammatory proteins detected in plasma EVs.

Protein Symbol	Protein Name
CCL19	C-C motif chemokine 19
CCL28	C-C motif chemokine ligand 28
CD244	Natural killer cell receptor 2B4
CD5	T-cell surface glycoprotein CD5
CD6	T-cell differentiation antigen CD6
CD8A	T-cell surface glycoprotein CD8 alpha chain
CD40	Cluster of differentiation 40
CSF-1	Macrophage colony-stimulating factor 1
CST5	Cystatin D
CXCL1	C-X-C motif chemokine ligand 1
CXCL5	C-X-C motif chemokine ligand 5
CXCL6	C-X-C motif chemokine ligand 6
CXCL11	C-X-C motif chemokine ligand 11
DNER	Delta and Notch-like epidermal growth factor related receptor
HGF	Hepatocyte growth factor
IL-1alpha	Interleukin-1 alpha
IL18	Interleukin-18
IL-18R1	Interleukin-18 receptor 1
LAPTGF-beta-1	Latency-assoc. peptide transforming growth factor b-1
MCP-1	Monocyte chemotactic protein 1
MCP-2	Monocyte chemotactic protein 2
MCP-4	Monocyte chemotactic protein 4
MMP-1	Matrix metalloproteinase-1
OPG	Osteoprotegerin
ST1A1	Sulfotransferase 1A1
STAMBP	STAM-binding protein
TRAIL	TNF-related apoptosis-inducing ligand
uPA	Urokinase-type plasminogen activator
VEGFA	Vascular endothelial growth factor A

Supplementary Table 3. Regression results for plasma mtDNA regions with perceived discrimination, race, sex, poverty status and age

Region	Poverty * Discrimination	Poverty * Age	Race * Age	Sex * Age
3164	0.012			
4625		0.042	0.041	
7878	0.023			
8446			0.034	0.045

Only significant p-values (<0.050) for the term indicated are displayed.

Supplementary Table 4. Regression results for EV mtDNA regions with perceived discrimination, race, sex, poverty status and age

Region	Poverty * Discrimination	Age
3164	0.042	
4625		
7878	0.003	
8446	0.019	0.044

Only significant p-values (<0.050) for the term indicated are displayed.

Supplementary Table 5. Regression results for EV inflammatory proteins with perceived discrimination, race, sex, poverty status and age

Inflammatory Proteins	Race * Discrimination	Sex * Discrimination	Age * Discrimination	Race * Sex	Race * Age	Sex * Agecen	Race	Age
CD6					0.016			0.021
CSF-1				0.025		0.011		
Cystatin D+	0.043	0.008			0.003	<0.001		
DNER						0.027		0.012
IL-18			0.021	0.016				
LAPTGF-beta-1				0.005				
OPG						0.047		
STAMBP							0.034	
TRAIL				0.039				

+ Values were positively skewed so transformed as log2(value+1). Only significant p-values (<0.050) for the term indicated are displayed

Supplementary Table 6. Logistic regression results for presence of EV inflammatory proteins with perceived discrimination, race, sex, poverty status and age

Inflammatory Proteins	Poverty * Discrimination	Sex * Discrimination	Sex * Age	Race * Age	Poverty * Age	Race * Sex	Poverty * Sex	Race	Age
CASP-8	0.020			0.026					
CCL23			0.049	0.037					
CCL25								0.003	
IL-8		0.048					0.014		
MCP-2									0.031
PD-L1					0.038	0.008			
TNF	0.021								
TNFSF-14								0.014	
TWEAK		0.018		0.002					

Only significant p-values (<0.050) for the term indicated are displayed.