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Extracellular Vesicle Mitochondrial DNA Levels Are Associated With Chronic Kidney Disease and Mitochondrial Haplogroup in Obese Individuals

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ABSTRACT

Chronic kidney disease (CKD) and obesity are major chronic diseases in the United States. Although obesity is a risk factor for CKD, little is known about how obesity contributes to CKD. Due to their role as intercellular communicators, extracellular vesicles (EVs) may be a factor connecting obesity and CKD. Circulating cell-free mitochondrial DNA (ccf-mtDNA), a damage-associated molecular pattern molecule associated with inflammation, is associated with renal disease and may be encapsulated within EVs. In this longitudinal study, we isolated plasma EVs and analysed EV mtDNA levels in a cohort of African American and White obese middle-aged individuals who at visit 1 did not have CKD but developed CKD by visit 2 ($n = 19$; CKD group) and matched this group to controls who did not develop CKD by visit 2 ($n = 56$; control group). In our cross-sectional analyses at visit 1, we found significant interactions for EV mtDNA levels between race and CKD status, poverty status and CKD status, and sex and CKD status. EV mtDNA levels were significantly lower in participants within the African haplogroup who developed CKD compared to participants within the European haplogroup who developed CKD and the African haplogroup control group. In our longitudinal analyses using data from both visit 1 and visit 2, individuals who developed CKD had lower EV mtDNA levels. Stratification by haplogroup showed that among participants within the African haplogroup, those who developed CKD had significantly lower EV mtDNA levels than those in the control group. In conclusion, EV mtDNA levels were lower in individuals who develop CKD. Our findings demonstrate that CKD status and mtDNA haplogroup influence EV cargo in obese individuals.

1 | Introduction

There is a high burden of both chronic kidney disease (CKD) and obesity in the United States (US) (CDC 2023). CKD is an age-associated chronic disease that affects 35.5 million adults in the US (CDC 2023). In addition, approximately 41.9% of adults in the US are obese (CDC 2024). While it is known that obesity is a risk factor for CKD (Chen et al. 2021), we do not fully

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understand how obesity contributes to CKD. Obese patients progress to end-stage renal disease (ESRD) faster than non-obese patients (Jacob and McCafferty 2023). The rate of ESRD increases directly proportionate to body mass index (BMI) (Hsu et al. 2006). Obesity is also associated with more proteinuria and enlarged glomeruli, which result in downstream health consequences such as renal insufficiency, hypertension and obstructive sleep apnoea (Jiang et al. 2023). Further understanding possible pathways of connection between obesity and CKD is especially important given the rise in obesity rates worldwide; 43% of adults are overweight and 16% are obese (World Health Organization 2024).

Due to their role as intercellular communicators, extracellular vesicles (EVs) may be candidates for investigating how obesity effects CKD. EVs are membranous structures that are released into the extracellular environment (van Niel et al. 2018; Yanez-Mo et al. 2015) and encompass a wide range of particles, including exosomes, microvesicles and apoptotic bodies (van Niel et al. 2018; Welsh et al. 2024; Witwer and Thery 2019). EVs have garnered attention due to their potential as disease biomarkers and the minimally invasive nature of collecting EVs from biofluids (Yanez-Mo et al. 2015; Yates et al. 2022). Previously, we reported that EVs can carry circulating cell-free mitochondrial DNA (ccf-mtDNA) as cargo (Lazo et al. 2021). Ccf-mtDNA is cellular mtDNA that is released into the extracellular space and can act as a damage-associated molecular pattern (DAMP) molecule. Upon cellular damage, stress, ischaemia, or trauma, cellular components including ccf-mtDNA, nuclear DNA, specific proteins and reactive oxygen species can be extruded into the extracellular space. DAMPs are recognized by specific pattern recognition receptors that then provoke downstream pro-inflammatory effects (Feldman et al. 2015; Grazioli and Pugin 2018; West et al. 2011).

There are links between mitochondrial function and kidney health. Renal tubular cells have a high density of mitochondria compared to other cells in the body (Irazabal et al. 2022), indicating that mitochondrial dysfunction can impact kidney diseases. Several studies have found an association between kidney dysfunction and mtDNA copy number, the number of copies of mtDNA per cell. Circulating mtDNA copy number is a relative or absolute number quantified based on different methods (Filograna et al. 2021). It can be derived by analysing a specific region of interest or by using genotyping arrays or sequencing technologies (Filograna et al. 2021). Studies in both human and rat tissues and cells have found an association between reduced mtDNA copy number and decreased mitochondrial oxidative phosphorylation (OXPHOS) capacity (Barazzoni et al. 2000; Czajka et al. 2015; Short et al. 2005). Thus, mtDNA copy number in blood has been proposed as a surrogate indicator of mitochondrial function in tissues, including the kidney (Malik 2023). A community-based cohort study found that participants with higher mtDNA copy number in their peripheral leukocytes had a decreased prevalence of microalbuminuria (Lee et al. 2009), while others found negative associations between mtDNA copy number in the blood and CKD incidence (Tin et al. 2016) and progression (He et al. 2022). Two studies calculated mtDNA copy number from peripheral blood (Lee et al. 2009; Tin et al. 2016), mainly peripheral blood mononuclear cells (PBMCs), while one study measured ccf-mtDNA in serum (He et al. 2022). The reasoning behind why individuals with kidney diseases have lower mtDNA copy number is still being investigated, but mitochondrial fragmentation is a plausible explanation. In the context of obesity, it was found that ccf-mtDNA levels increase as BMI increases in adults over the age of 50 (Padilla-Sanchez et al. 2020). However, the studies on CKD and obesity were performed by analysing mtDNA levels in whole serum or in PBMCs and did not examine the EV-associated ccf-mtDNA fraction in plasma/serum. Thus, we are only beginning to understand the different pools of ccf-mtDNA in plasma/serum and the mechanisms that contribute to these subsets in plasma in general (Trumpff et al. 2021) and in the context of obesity and CKD.

Mutations in mtDNA amass throughout maternal lineage, allowing specific single nucleotide polymorphism (SNP) combinations to be identified and traced to geographic origins of populations (Birky 1995; Wei and Chinnery 2020). These SNP combinations delineate mtDNA haplogroups (Cann et al. 1987; Chen et al. 1995; van Oven and Kayser 2009). Cytoplasmic hybrid (cybrid) studies have illuminated how different mtDNA haplogroups have varying levels of reactive oxygen species (ROS), mtDNA copy number, oxidative phosphorylation capacity, growth rates and mtDNA damage (Chen et al. 2012; Gomez-Duran et al. 2010; Kenney et al. 2014; Krzywanski et al. 2016). Therefore, mtDNA haplogroup can affect mitochondrial function (Wallace 2015). We previously reported that EV mtDNA levels were associated with mtDNA haplogroup in a cohort of normotensive and hypertensive individuals (Byappanahalli et al. 2024).

Emerging evidence suggests that circulating EVs hold promise as biomarkers of CKD (Georgatzakou et al. 2020) and obesity (Noren Hooten and Evans 2020; Wang and Zeng 2023). However, the majority of the studies have focused on larger circulating vesicles detected by flow cytometry (Georgatzakou et al. 2020; Noren Hooten and Evans 2020). In obesity, adipocyte dysfunction can cause inflammation and fibrosis (Unamuno et al. 2018), and EVs may be key facilitators in this process (Wang and Zeng 2023). Therefore, EVs may link obesity and CKD. In this longitudinal study, we investigated whether EV mtDNA differs between obese individuals with and without CKD in a cohort of African American and White adults.

2 | Methods

2.1 | Human Participants

The study cohort was from the HANDLS study conducted by the National Institute on Aging Intramural Research Program, National Institutes of Health (NIH) (Evans et al. 2010). Participants self-reported race, and poverty status was determined by household income at enrolment defined as above or below 125% of the 2004 US Health and Human Services Poverty Guidelines (HHS 2004). All participants gave written informed consent. The Institutional Review Board of the NIH approved HANDLS.

The initial recruitment of the HANDLS study included 3720 participants. The cohort design for this study is shown in a flowchart (Figure S1). The following inclusion criteria were used for participants: (1) had stored blood samples from two visits; (2) had a BMI above 25 kg/m^2 at both visits (includes both overweight and obese participants, hereafter referred to as obese); (3) no

TABLE 1 | Study cohort.

Subcohort	CKD group ($N = 19$)	Control group ($N = 56$)	p value
Men, N (%)	8 (42)	24 (43)	1.000
African American, N (%)	11 (58)	32 (57)	1.000
Below Poverty, N (%)	10 (53)	29 (52)	1.000
Age at time 1*, mean (SD)	56.9 (7.8)	52.5 (6.0)	0.022
Haplogroup			0.442
African, N (%)	11 (58)	31 (55)	
European, N (%)	6 (32)	23 (41)	
Other, N (%)	2 (11)	2 (4)	
Data by time point	**Visit 1 ($N = 61$)**	**Visit 2 ($N = 74$)**	**p value**
Age, mean (SD)	53.7 (6.9)	58.3 (7.0)	< 0.001
BMI, mean (SD)	32.9 (6.3)	33.3 (6.4)	0.712
BMI overweight, N (%)	28 (46)	27 (37)	0.351
BMI obese, N (%)	33 (54)	47 (63)	
eGFR, mean (SD)	89.8 (14.6)	77.2 (20.1)	< 0.001

Note: Tests of differences for categorical variables used the Pearson Chi-squared test. Tests of differences for continuous variables used a Student's t-test or, for two time points, a paired Student's t-test. BMI categories are overweight (25–29.9 kg/m^2) and obese (> 30 kg/m^2). Abbreviations: BMI, body mass index; CKD, chronic kidney disease; eGFR, estimated glomerular filtration rate; SD, standard deviation.
*Sample size at visit 1 for those with samples ($N = 61$).

CKD at visit 1. Participants with diabetes mellitus were excluded. CKD was defined as an estimated glomerular filtration rate (eGFR) below 60 mL/min/1.73 m^2 (Inker et al. 2021) using a race-independent measure based on cystatin C and serum creatinine levels (2021 CKD-EPI creatinine-cystatin C (Inker et al. 2021)). This eGFR equation has higher estimation accuracy than other formulae and eliminates bias from the race correction previously used in the eGFR algorithm. Using these criteria, we selected 19 HANDLS participants who did not have CKD at their first visit and had developed CKD by their second visit (Figure 1A; Table 1) (referred to as the CKD group). A control group without CKD at both visits (referred to as the control group) was matched to the CKD group by race, sex, poverty status and median age split, with three controls for every one CKD group participant. Due to a lack of adequate blood samples, some participants only had samples from one visit. The final cohort consisted of 135 samples from 75 participants (CKD group = 19, control group = 56) over two visits (visit 1 = 61, visit 2 = 74; Table 1).

2.2 | Plasma EV Isolation

Plasma was isolated from fasting participants as described in detail in the Supplemental Methods according to MIBlood-EV guidelines (Lucien et al. 2023) and were separated into fractions via size exclusion chromatography with the Automatic Fraction Collector (IZON, Cat: AFC-V2) using the qEV Original 70 nm Gen2 column (IZON, Cat:ICO70). Ten (0.5 mL) fractions were collected, and the buffer volume for each collection was set to 2.5 mL. Columns were flushed with 17 mL of 1X filtered phosphate buffered saline (PBS) prior to sample loading. Plasma sample (0.4 mL) was loaded, 8 mL of 1X filtered PBS was added to the column, and then fractions were collected. Columns were cleaned with 8.5 mL of 0.5 M NaOH and flushed with 17 mL of 1X filtered PBS. Each column was reused 5 times, based on the manufacturer's recommendations. Fractions 1–3 were considered the EV-enriched fraction and were combined and aliquoted as follows: whole EVs (300 μL), DNA isolation (155 μL), diluted 1:5 in 1X PBS for NTA (100 μL) and the remaining volume was concentrated and lysed. Aliquots were stored at −80°C until use.

2.3 | Immunoblotting

EV-enriched fractions (F1–3; 945 μL) were concentrated using an Amicon Ultra 10 kDa-0.5 mL Centrifugal Filter Unit (Cat: UFC501008), according to the manufacturer's protocol. The final concentrated sample was lysed in a 10X lysis buffer as previously described (Byappanahalli et al. 2023).

The concentrated EV lysates and human umbilical vein endothelial cell (HUVEC) lysate were run on 4%–12% Bolt Bis-Tris Plus gels (Invitrogen Thermo Fisher Scientific), transferred to PVDF, and then immunoblotted with antibodies (diluted 1:500) against CD9 (System Biosciences, Cat: EXOAB-CD9A-1), GM130 (Abcam, Cat: AB52649) and Flotillin-1 (Abcam, Cat: AB133497). After incubation with secondary horseradish peroxidase-conjugated antibodies (Cytiva Life Sciences, Cat: NA934V), blots were imaged using a ChemiDoc MP Imaging System (Bio Rad) with the Kwik-Quant Ultra HRP Substrate solution (Kindle Biosciences, LLC; Cat: R1002).

2.4 | Nanoparticle Tracking Analysis

SEC isolated EVs were diluted as described above and analysed using a NanoSight NS500 (Malvern Panalytical, software version NTA 3.4 Build 3.4.4). Samples were further diluted if necessary, and this was accounted for when calculating particle

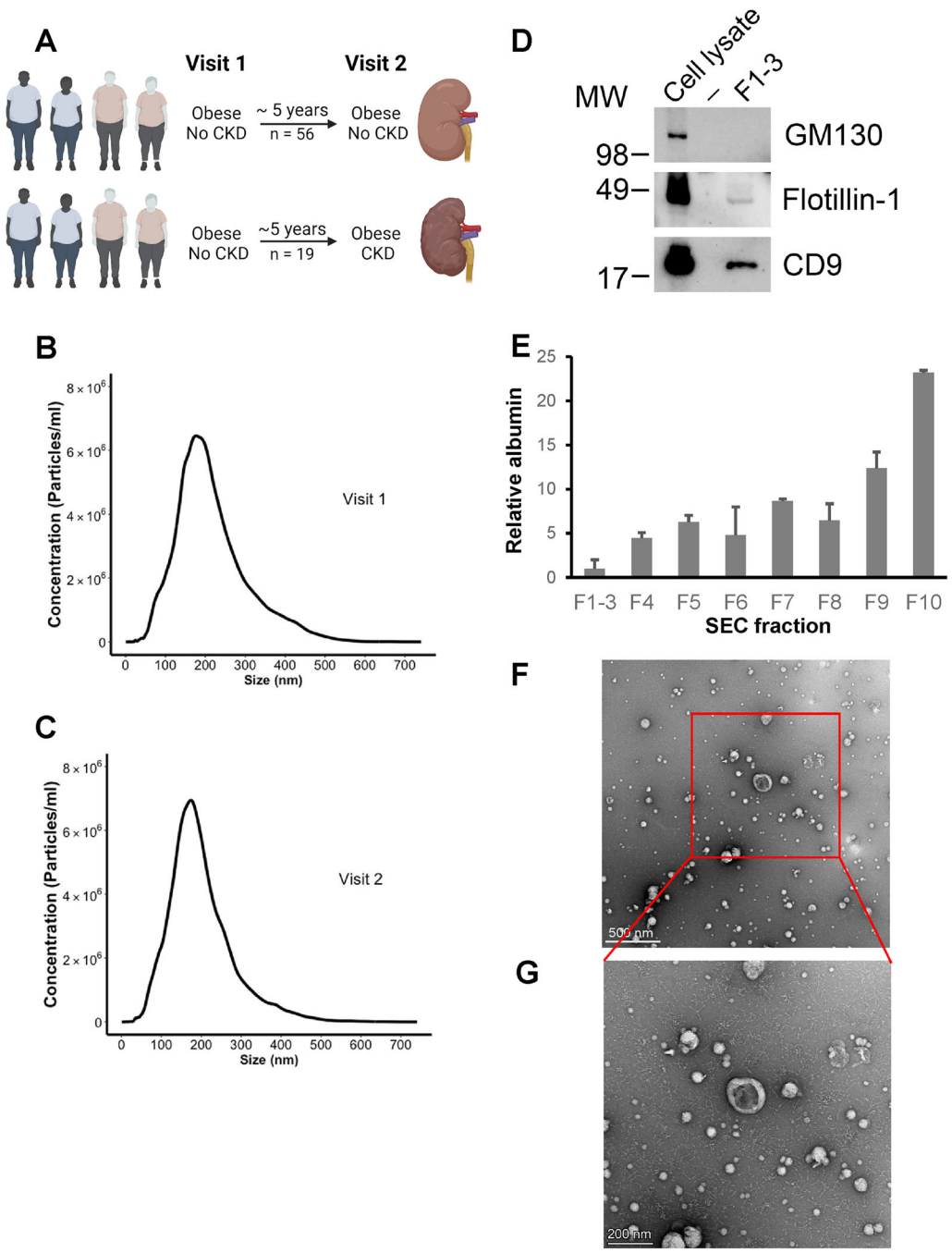

FIGURE 1 | Plasma EV characteristics of CKD cohort. (A) Schematic of cohort design. Plasma EV concentration and size distribution were analysed via nanoparticle tracking analysis. The size distribution was averaged for (B) visit 1 ($n = 61$) and (C) visit 2 ($n = 74$). (D) Plasma EVs (F1–3) and HUVECs were lysed and immunoblotted with antibodies against known EV markers CD9, and Flotillin-1. GM130 served as a negative control. (E) Size exclusion chromatography (SEC) fractions were analysed for albumin. F1–3 are the pooled extracellular enriched fractions. The histogram represents the relative mean albumin concentration for fractions from participants from the CKD cohort ($n = 3$) + standard error of the mean. (F)–(G) EV morphology was visualized with electron microscopy. A representative low (F) and high (G) magnification image are shown. The region outlined in red is shown in the high magnification image ([F] scale bar = 500 nm; [G] scale bar = 2-G00 nm). CKD = chronic kidney disease, F = fraction, SEC = size exclusion chromatography.

concentration. Each sample was recorded in 5 videos, 20 s each (camera level = 16 and detection level = 3). All samples were run on the same machine by one user. Total EV concentration of each sample was calculated as described (Eitan et al. 2017). EV concentration values were positively skewed and therefore were $\log_2$ transformed for analysis.

2.5 | Assessment of Albumin Levels

Albumin concentration was quantified from all SEC fractions collected from the plasma of three participants from the CKD cohort using an albumin ELISA kit (Abcam; cat. no. ab179887). SEC fractions were equally diluted (1:400,000), and analyte

concentration was extrapolated from raw absorbances using the trendline equations of calibration curves. Sample absorbance at 450 nm was read on a SpectraMax M2 Microplate Reader (Molecular Devices, LLC). Samples with signals below the limit of detection were imputed a value of 0. For each sample, the fractions were normalized to the mean of the EV fractions (F1–3).

2.6 | EV DNA Isolation and Quantitative Real-Time PCR

Equal volumes of SEC isolated EVs (155 μL) were used for DNA isolation. EVs were DNase treated, and DNA was isolated using the DNeasy Blood and Tissue kit (Qiagen, Cat: 69506) as described (Byappanahalli et al. 2023; Lazo et al. 2021). For plasma DNA isolation, whole plasma was thawed on ice, and 25 μL of sample was added to nuclease-free water (175 μL) for a final volume of 200 μL. DNA was isolated using the DNeasy Blood and Tissue kit, with the exception that the DNase treatment step was omitted. Quantitative real-time PCR (qPCR) was conducted using four mtDNA primers, as previously designed and described (Lazo et al. 2021). Each reaction consisted of 3 μL EV DNA, 5 μL Taqman Fast Advanced Master Mix, 0.8 μL primers, and 1.2 μL nuclease-free water. For qPCR for plasma DNA, the reactions consisted of 4.2 μL EV DNA, 5 μL Taqman Fast Advanced Master Mix, 0.8 μL primers. Samples were run on a 384-well plate using the ViiA 7 System (QuantStudio Real-Time PCR Software v1.6.1). mtDNA levels from each mtDNA primer set were calculated using a derivation of the $2^{-\Delta\Delta Ct}$ method. For each mtDNA primer set, the Ct value for each sample was normalized to the global mean (X; mean of the Ct for each primer set). Thus, the following formula was used $2^{-(X-Ct)}$, as described in detail previously (Lazo et al. 2021). EV and plasma mtDNA values were positively skewed and therefore were $\log_2$ transformed for analysis.

2.7 | Electron Microscopy

Electron microscopy was conducted by the Johns Hopkins University School of Medicine Microscope Facility. Plasma EVs were freshly isolated and absorbed to freshly ionized 400 mesh formvar/carbon coated grids (Electron Microscopy Sciences, Cat: CF400-Cu-UL) for 2 mins and then washed with 3 drops of Tris-buffered saline. Samples were negatively stained in 1% aqueous uranyl acetate. After loading the sample onto the grid, image areas were randomly selected to capture a representative image that shows the approximate density of the sample with good contrast and a low background. Images were acquired with a transmission electron microscope (Thermo Fisher Talos L120C) at 120 kV and a Thermo Fisher Ceta CCD (16-megapixel CMOS, 16-bit) camera. A representative low- and higher-magnification image was chosen that shows the breadth of EVs that were visualized.

2.8 | Determination of Mitochondrial DNA Haplogroups

mtDNA haplogroups were determined for each participant after conducting allelic discrimination PCR assays, which were completed by the Department of Ophthalmology Research at the University of California, Irvine. Ten allelic discrimination markers were used for qPCR, and haplogroups were determined as previously described (Byappanahalli et al. 2024). Participants were assigned mtDNA haplogroups and then grouped into African (L0, 1, 2, 4, 5, 5, and L3), European (H, HV, J, K, T, U and UK), or Other (B-P-F-R, M, and N-A-Y-W-I-X) mtDNA ancestry groups.

2.9 | Statistical Analysis

Statistical analysis was conducted using R software version 4.3.2 (R Core Team 2023). Tests of differences for categorical variables used the Pearson Chi-squared test. Tests of differences for continuous variables used a Student's *t*-test or, for two time points paired Student's *t*-test. Variables of interest were assessed for skewness; those with skewness values greater than 2 were $\log_2$ transformed as noted in each section above. Pearson correlations were calculated between the EV mtDNA regions of interest. Cross-sectional analyses (visit 1) used multivariable linear regression with backward elimination starting with all two-way interactions. Non-significant interactions were removed. Longitudinal analyses (visits 1 and 2) used linear mixed model regression accounting for the repeated measurements over time with backward stepwise elimination. Models analysing EV mtDNA levels, EV concentration and EV mean size included race, sex, CKD status, poverty status and age. Models analysing mtDNA haplogroup status included mtDNA haplogroup, sex, CKD status, poverty status and age in the model. A p value < 0.05 was used for statistical significance, and adjustments for multiple comparisons were not performed due to the small sample size.

3 | Results

3.1 | Plasma EV Characteristics of CKD Cohort

To determine if CKD alters EV-associated cargo, we chose a cohort of obese middle-aged participants from the HANDLS study who at visit 1 did not have CKD and who developed CKD by visit 2 ($n = 19$; CKD group). We matched this group to controls who did not develop CKD by visit 2 ($n = 56$; control group) (Figure 1A; Table 1). Plasma EVs from participants were isolated using size exclusion chromatography. Minimal Information for Studies of Extracellular Vesicles (MISEV) guidelines were used to validate the isolated plasma EVs (Welsh et al. 2024). Nanoparticle tracking analysis (NTA) was used to validate the size and concentration of the isolated EVs from visit 1 and visit 2 (Figures 1B–C). The size distribution for the EVs from this cohort peaked around 200 nm (Figures 1B–C), which is typical of plasma-derived EVs. Immunoblotting confirmed the presence of EV-associated markers. Positive EV markers Flotillin-1 and CD9 were present in EV-enriched fractions F1–3, while the purity marker GM130 was absent (Figure 1D). We also analysed co-contaminants by measuring the fractionation of albumin, one of the most abundant free proteins in human blood plasma. We quantified albumin levels in intact SEC fractions using an ELISA assay from three different samples from our CKD cohort. The results showed that our EV-enriched fraction (F1–3) had the lowest amount of albumin and that there was a gradual increase of albumin in parallel with plasma fractionation (Figure 1E). The results suggest that the EV-enriched fraction

(F1–3) is purified from free plasma proteins. Electron microscopy of the EVs showed typical EV morphology characterized by round membranous vesicles, with some vesicles exhibiting a cup-like shape commonly caused by the dehydration process in imaging sample preparation (Figure s3 1F-G). Linear regression models examined if there were significant differences in EV size or EV concentration at visit 1 related to the variables of interest: race, sex, CKD status, poverty status, and age. No significant differences were found.

3.2 | EV mtDNA Levels at Visit 1 Are Associated With Race, Sex, Poverty Status and CKD Group

Ccf-mtDNA is a DAMP molecule that can be found as plasma EV-encapsulated cargo (Lazo et al. 2021; Noren Hooten and Evans 2021). Initially, we wanted to compare the levels of ccf-mtDNA in the SEC fractions. Plasma was pooled from four individuals, and plasma was fractionated into 10 fractions using SEC. Fractions 1–3 (F1–3) were combined for the EV-enriched fraction. DNA was isolated from equal parts of SEC fractions, and mtDNA levels were quantified by qPCR using four primer sets targeting different regions of the mitochondrial genome: 16S rRNA (*MT-RNR2*) and tRNA-Ile1 (*MT-TL1*) genes (Mito_3164), the NADH dehydrogenase 2 (*MT-ND2*) gene region (Mito_4625), Cytochrome c oxidase subunit 2 (*COX2*) gene region (Mito_7878), and the ATP6/8 (*MT-ATP8*) gene region (Mito_8446) (Figure 2A). The EV-enriched fraction (F1–3) had significantly higher mtDNA levels than Fractions 4, 5 or 6 (Figure S2).

Using this knowledge, we analysed EV-associated ccf-mtDNA levels in our longitudinal cohort using a previously described experimental workflow (Byappanahalli et al. 2023; Lazo et al. 2021). Plasma EVs were treated with DNase to degrade any unencapsulated DNA outside of the EVs, EV DNA was isolated, and qPCR was used to quantify EV mtDNA. Pearson correlation was used to determine the relationship between EV mtDNA levels amplified with the four primer sets. All four mtDNA amplicons were positively correlated with one another, and the correlations were all statistically significant (Figure S3). Therefore, all four regions of the mitochondrial genome were present in the EVs and were strongly correlated with each other.

Linear regression was used to analyse the cross-sectional associations between EV mtDNA levels and CKD status at visit 1, accounting for race, sex, age, and poverty status. All results from the linear regression analysis are reported in Table S1. Only significant findings are visualized and described in detail and reported in Figures 2–3. We found significant interactions for EV mtDNA levels between CKD status and race ($p = 0.020$) (Figure 2B), CKD status and poverty status ($p = 0.019$) (Figure 2C), and CKD status and sex ($p = 0.040$) (Figure 2D). For Mito_3164, EV mtDNA levels among African American participants were significantly lower for those who will develop CKD than those in the control group. Among those who will develop CKD, African American participants had lower Mito_3164 levels than White participants (Figure 2B). Among participants living above poverty, those who will develop CKD had lower Mito_3164 EV mtDNA levels than those in the control group (Figure 2C). For Mito_8446, among those who will develop CKD, women had significantly higher levels of EV mtDNA than men. Men who will develop CKD had significantly lower EV mtDNA levels than control group men (Figure 2D).

3.3 | EV mtDNA Levels at Visit 1 Are Associated With Mitochondrial Haplogroup and CKD Group

Since we previously reported differences in EV mtDNA levels with ancestry and race (Byappanahalli et al. 2024), we sought to analyse the relationship between EV mtDNA and ancestry in this cohort. Cohort participants were haplotyped and assigned a haplogroup of African, European, or Other (Table 1). There was a high concordance between reported race and mtDNA haplogroup: 95% (41/43) of the African American participants had the African haplogroup, 84% (27/32) of White participants had the European haplogroup, and 91% of all participants had a reported race and haplogroup concordance (68/75). Participants assigned the Other haplogroup ($n = 4$) were excluded from all haplogroup analyses.

Linear regression was used to analyse the cross-sectional associations between EV mtDNA levels and CKD group at visit 1, accounting for haplogroup, sex, age, and poverty status. Significant interactions between CKD status and haplogroup were found (Figures 3A–C). EV mtDNA levels amplified by the Mito_3164, Mito_4625 and Mito_7878 primer sets were significantly lower in participants within the African haplogroup who developed CKD than participants within the European haplogroup who developed CKD (Figures 3A–C). Among participants in the African haplogroup, those who will develop CKD had significantly lower EV mtDNA levels than the control group. These data indicate that ancestry and CKD status are associated with EV mtDNA levels.

3.4 | EV mtDNA Levels Are Associated With CKD Status Longitudinally

To determine whether the development of CKD was associated with changes in EV mtDNA levels, we examined EV mtDNA in our longitudinal cohort using data from both visit 1 and visit 2. Results from the linear regression analysis are reported in Table S1. Only significant findings are visualized and described in detail and reported in Figures 4–5. Participants who developed CKD had significantly lower Mito_8446 EV mtDNA levels ($p = 0.011$) compared with the control group (Figure 4). Thus, individuals who develop CKD have lower EV mtDNA levels. There were no significant differences or interactions among our variables of interest for EV concentration, size or other mtDNA regions in our longitudinal analysis.

3.5 | Longitudinal EV mtDNA Levels Are Associated With CKD and mtDNA Haplogroup

Next, we used mtDNA haplogroup to analyse the relationship between ancestry and EV mtDNA levels longitudinally. We found that the CKD group was a significant main effect in our longitudinal analysis with haplogroup and Mito_8446 ($p = 0.021$). We then stratified the analysis by haplogroup to see if the results were similar in Mito_8446 EV levels for participants in

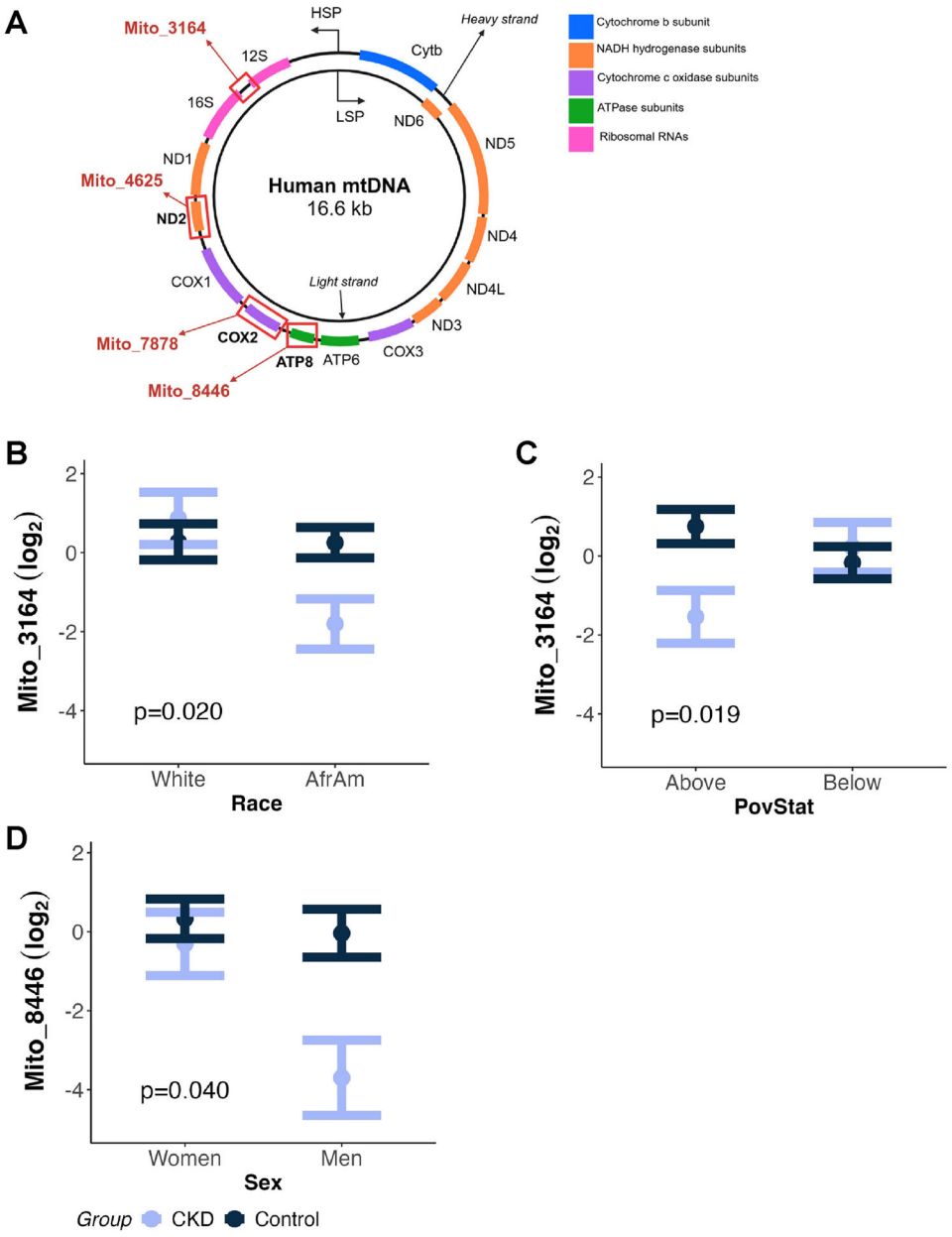

FIGURE 2 | EV mtDNA levels at visit 1 are associated with race, poverty status, sex, and CKD status. (A) mtDNA primers schematic. Mitochondrial genome with mtDNA primer regions indicated with a red box and arrow denoting primer name by starting nucleotide. Created in BioRender. N. Noren Hooten (2025) https://BioRender.com/o35e228. Linear regression was used to analyse cross-sectional associations between EV mtDNA levels (log_2 transformed) and CKD status, accounting for race, sex, poverty, and age. Significant interactions between (B) race and CKD, (C) poverty and CKD, and (D) sex and CKD are shown. The plots show the regression values ± standard error of estimated values. AfrAm = African American.

the African versus European haplogroups. Among participants within the African haplogroup, those who developed CKD had significantly lower EV mtDNA levels than those in the control group ($p = 0.014$) (Figure 5A). This relationship was specific to those with the African haplogroup, as there were no significant differences with EV mtDNA levels and the CKD group in those with the European haplogroup. We also found that for participants within the African haplogroup, Mito_8446 EV mtDNA levels increased with age ($p = 0.023$) (Figure 5B). Among participants within the European haplogroup, women had significantly higher Mito_8446 EV mtDNA levels than men ($p = 0.027$) (Figure 5C).

3.6 | Plasma mtDNA Levels in the CKD Cohort

There are several forms of ccf-mtDNA that can exist in the circulation, but little is known about the different biological forms of ccf-mtDNA in blood or the relationships between the different forms (Trumpff et al. 2021). To address this question, we examined ccf-mtDNA in whole plasma in our CKD cohort. DNA was isolated from plasma, and ccf-mtDNA levels were quantified using the four different mitochondrial genome primer sets (Figure 2A). Using Pearson correlation, we initially examined the relationship between plasma and EV mtDNA levels. There was a significant correlation between plasma and EV

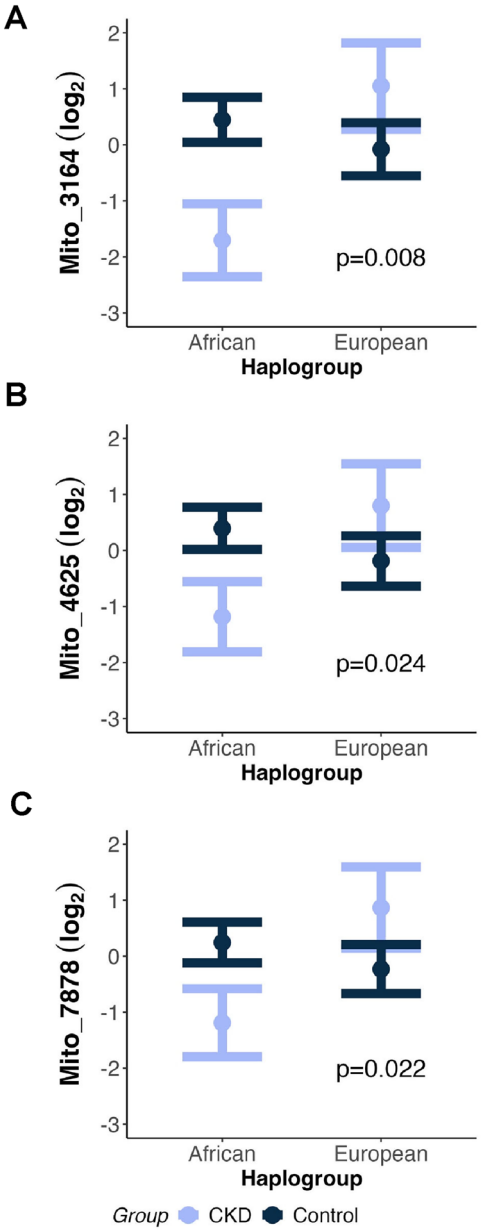

FIGURE 3 | EV mtDNA levels at visit 1 are associated with haplogroup and CKD status. Linear regression was used to analyse cross-sectional associations between EV mtDNA (log_2 transformed) levels and CKD status, accounting for haplogroup, sex, poverty status, and age. Significant interactions between haplogroup and CKD for the (A) Mito_3164, (B) Mito_4625, and (C) Mito_7878 primer sets are shown. The plot shows the regression values ± standard error of estimated values.

mtDNA levels for each mtDNA region (Figure S4). We next used linear regression to analyse the relationship of plasma mtDNA with race, sex, CKD group, poverty status, and age using our cross-sectional and longitudinal data. In our cross-sectional analysis, we observed significant interactions with different plasma mtDNA levels with CKD status and race; CKD status and poverty; and CKD status and age (Table S2). In our models with haplogroup, plasma mtDNA was associated with CKD status and haplogroup and also with CKD status and age. In our longitudinal analysis, there were significant interactions of Mito_8446 with CKD status and race; CKD status and age;

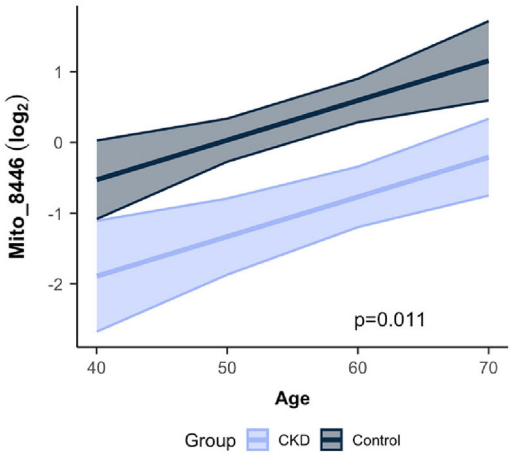

FIGURE 4 | Longitudinal EV mtDNA levels are lower in participants who developed CKD. Linear mixed model regression was used to analyse longitudinal associations between EV mtDNA levels (log_2 transformed) and CKD status, accounting for race, sex, poverty status, and age. The plot shows the regression values ± standard error of estimated values.

and race and age. In the models with haplogroup, there were significant interactions of Mito_8446 levels with CKD status and race and also with CKD and age. Results from the cross-sectional and longitudinal analyses are presented in Table S2. Overall, the data comparing the relationships between plasma and EV mtDNA levels with our variables of interest are complex, and some relationships are consistent, whereas many relationships were not consistent between the two different pools of ccf-mtDNA. This possibly indicates different mechanisms underlying release into the circulation and/or different functions for these forms of ccf-mtDNA in the circulation.

4 | Discussion

In this longitudinal study, we examined plasma EVs from African American and White obese adults who developed CKD compared with a matched control group. This study is unique as it yielded both cross-sectional and longitudinal results. In our cross-sectional analysis at visit 1, we found that EV mtDNA levels were associated with future CKD status through separate interactions with race, poverty and sex. Thus, EV mtDNA levels are influenced differentially by future CKD status across sex, poverty and race in this cohort of obese adults. We also observed cross-sectional differences in EV mtDNA levels with future CKD status and mtDNA haplogroup. In our longitudinal analysis, EV mtDNA levels were lower in participants who developed CKD only for those in the African haplogroup. Furthermore, EV mtDNA levels were associated with age and sex in the African and European mtDNA haplogroups, respectively.

Our findings that individuals with CKD or who will develop CKD have lower EV mtDNA levels is consistent with previous reports of lower ccf-mtDNA levels in patients with renal dysfunction, including CKD (He et al. 2022; Lee et al. 2009; Tin et al. 2016). In these studies, mtDNA copy number was measured in serum (He et al. 2022) or in whole blood, containing PBMCs (Lee et al. 2009; Tin et al. 2016). Therefore, our studies build upon these initial findings by identifying that ccf-mtDNA carried in EVs is lower in

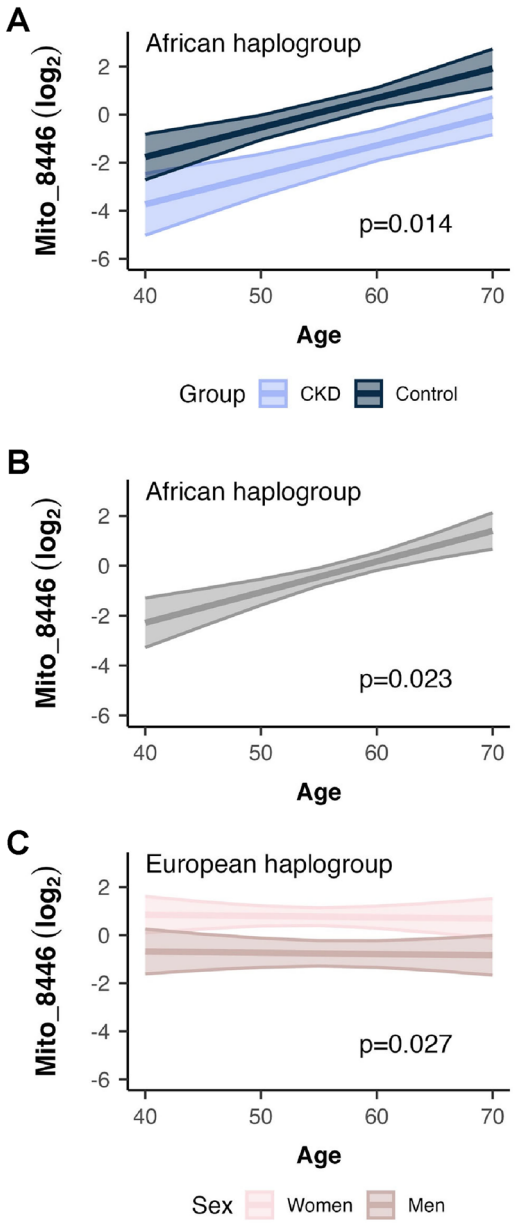

FIGURE 5 | Longitudinal EV mtDNA levels depend on mtDNA haplogroup for CKD status, age, and sex. Linear mixed model regression was used to analyse longitudinal associations between EV mtDNA levels (log_2 transformed) and CKD status, accounting for race, sex, poverty, and age. Among participants within the African haplogroup, (A) those who developed CKD had lower EV mtDNA levels than those who didn't develop CKD, and (B) EV mtDNA levels increased with age. (C) Among participants within the European haplogroup, women had higher EV mtDNA levels than men.

individuals with CKD. It is suggested that lower ccf-mtDNA levels may be an indicator of reduced renal function (Malik 2023). Thus, our data is consistent with this idea.

Not only did we examine ccf-mtDNA in EVs, but also in plasma. As some findings, but not all, were consistent between plasma and EV mtDNA levels, these data indicate that different mechanisms may underlie the release of these forms of ccf-mtDNA. In the circulation, mtDNA can be packaged in EVs (Lazo et al. 2021; Sansone et al. 2017), whole mitochondria (Al Amir Dache et al. 2020; Song et al. 2020; Stephens et al. 2020), protein-bound or potentially free-floating (Trumpff et al. 2021). Mitochondria are stress mediators that sense, integrate, and respond to stress. Stress or damage to mitochondria can result in the release of ccf-mtDNA, which can occur through a passive process of cell death or an active process via the mitophagy pathway (Al Amir Dache and Thierry 2023; Ferrucci et al. 2024; Trumpff et al. 2021). During mitophagy, damaged whole mitochondria or fragments can also be encapsulated into 'mitochondria-derived EVs (MDVs),' which are then encapsulated into multivesicular bodies (MVBs). These MVBs are then disposed of via the lysosomal/peroxisomal degradation pathways or secreted into the extracellular environment (Ferrucci et al. 2024). This release of MDVs has recently been described for cancer cells in the interstitial fluid and termed 'secretory mitophagy' (Howard et al. 2022). We are only beginning to understand if this is a general mechanism for the release of EVs containing mitochondrial components. Additional mechanisms whereby MDVs and mtDNA are released are actively being investigated (Al Amir Dache and Thierry 2023; Ferrucci et al. 2024; Trumpff et al. 2021). Recently, a subpopulation of MDVs termed 'mitovesicles' were described as EVs containing specific mitochondrial components from brain tissue (D'Acunzo et al. 2021). In our study, we analysed plasma EVs, which are heterogeneous in origin. Therefore, we do not know at this time the proportion of this population that may be 'mitovesicles' or 'MDVs.' In addition, although we believe that our SEC preparations contain mainly EVs and not any whole mitochondria, it is difficult to exclude the possibility that there may be circulating mitochondria or their parts that contain mtDNA in our preparations.

In our cross-sectional analysis at visit 1 and in our longitudinal analysis, CKD group participants in the African haplogroup had the lowest EV mtDNA levels. Very few studies have examined the role of mtDNA haplogroup in CKD (Cañadas-Garre et al. 2024; Guo et al. 2021; Jotwani et al. 2024; Yonova-Doing et al. 2021). These studies have primarily been conducted using European haplogroups (Cañadas-Garre et al. 2024; Jotwani et al. 2024; Yonova-Doing et al. 2021) or a Chinese haplogroup (Guo et al. 2021). These data suggest that SNPs in mtDNA and different haplogroups are associated with markers of renal function or kidney disease in European and Chinese individuals (Cañadas-Garre et al. 2024; Guo et al. 2021; Jotwani et al. 2024; Yonova-Doing et al. 2021). Thus far, there is limited knowledge about African haplogroups and CKD. One study examined cybrids from cells with haplogroup L (African haplogroup) compared to haplogroup J and H (European haplogroups) and found differences in ATP production and mtDNA damage (Krzywanski et al. 2016). Therefore, we are only beginning to understand the contribution of mtDNA haplogroups to CKD, especially with respect to the African haplogroup.

In our cross-sectional analysis, there were differences in Mito_3164 EV mtDNA levels with poverty and CKD status. Those living above poverty who will develop CKD had lower Mito_3164 EV mtDNA levels than those in the control group (Figure 2B). This finding is consistent with previous work in the Atherosclerosis Risk in Communities Study where investigators found that CKD was associated with lower mtDNA copy number, highlighting that mtDNA may be an important marker of CKD and CKD risk (Tin et al. 2016). We found that EV mtDNA levels

were similar for those living below poverty, regardless of CKD group. It is well-established that living in poverty is associated with CKD (Crews and Novick 2019). This greater burden of CKD among those living in poverty is multi-factorial in nature. In the HANDLS study, we previously reported that living below poverty had a significant relationship with CKD in African American adults, but not White adults (Crews et al. 2010). Therefore, it could be expected that there would be lower EV mtDNA levels in CKD participants living below poverty, which would be possibly reflective of reduced renal mitochondrial function. This is not what we found. It should be noted that for the HANDLS participants living above the 125% poverty line at baseline (2004–2007) only ~25% had an annual household income of > $50,000. The Baltimore median household income at that time was $44,262 (Asante-Muhammad 2017). Therefore, there is a complex interaction of social and biological factors that may affect the interaction of EV mtDNA levels with poverty status and CKD.

In our longitudinal results we found that our primer set Mito_8446, which encompasses the *MT-ATP8* gene, was lower in participants in the CKD group. This mtDNA gene encodes ATP8, a subunit of the ATP synthase (Complex V), which synthesizes ATP from ADP in oxidative phosphorylation and cellular energy production. Variants and alterations in *MT-ATP8* have been associated with mitochondrial dysfunction leading to various mitochondrial disorders (Del Dotto et al. 2024), including clinical phenotypes of renal insufficiency and disease (Del Dotto et al. 2024). Therefore, the *MT-ATP8* gene has been linked to mitochondrial defects and renal dysfunction.

Findings of this study should be examined with limitations in mind. The sample size for this study is similar to that of other EV-focused studies (Eitan et al. 2017; Lazo et al. 2021; Noren Hooten, Byappanahalli, et al. 2022), but may be too small to fully analyse the association of mtDNA haplogroup and CKD. Because CKD is most common in adults 65 years of age and older, the age of our cohort may be a limitation. Some participants may be younger than the typical age of CKD development by visit 2. However, individuals living in poverty may experience accelerated ageing, potentiating the onset of age-related illnesses and conditions earlier in life (Noren Hooten, Pacheco, et al. 2022). As plasma EVs are heterogeneous, different EV subpopulations may differ in mtDNA levels. EV isolation methods are a challenge in the field. In this study we used SEC to isolate EVs from plasma, which substantially removed soluble plasma proteins (Figure 1E). Although SEC can effectively separate EVs by size from non-vesicular extracellular particles (NVEPs), we cannot exclude that there may be NVEPs that may co-precipitate during the isolation process. Strengths of our study include having both African American and White participants as well as investigating the role of race and genetic ancestry through mtDNA haplogroups.

Our work indicates that EVs and their associated cargo may be clinical biomarkers for CKD. Obesity and CKD are chronic diseases that have been linked to accelerated ageing; thus, understanding these relationships could lend critical insights to ageing processes. As different population groups have premature morbidity and mortality from CKD (Hill et al. 2015), this project sheds light on important factors, such as ancestry, that should be considered in biomarker research.

Author Contributions

Jaida E. Morgan: formal analysis, investigation, methodology, writing – original draft. **Nicole Noren Hooten**: conceptualization, methodology, supervision, writing – original draft, writing – review and editing. **Nicolle A. Mode**: data curation, formal analysis, investigation, writing – review and editing. **Ngozi Ezike**: investigation, methodology, writing – review and editing. **Alan B. Zonderman**: data curation, formal analysis, funding acquisition, methodology, project administration, writing – review and editing. **Michele K. Evans**: conceptualization, funding acquisition, investigation, project administration, resources, supervision, writing – review and editing

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

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/.

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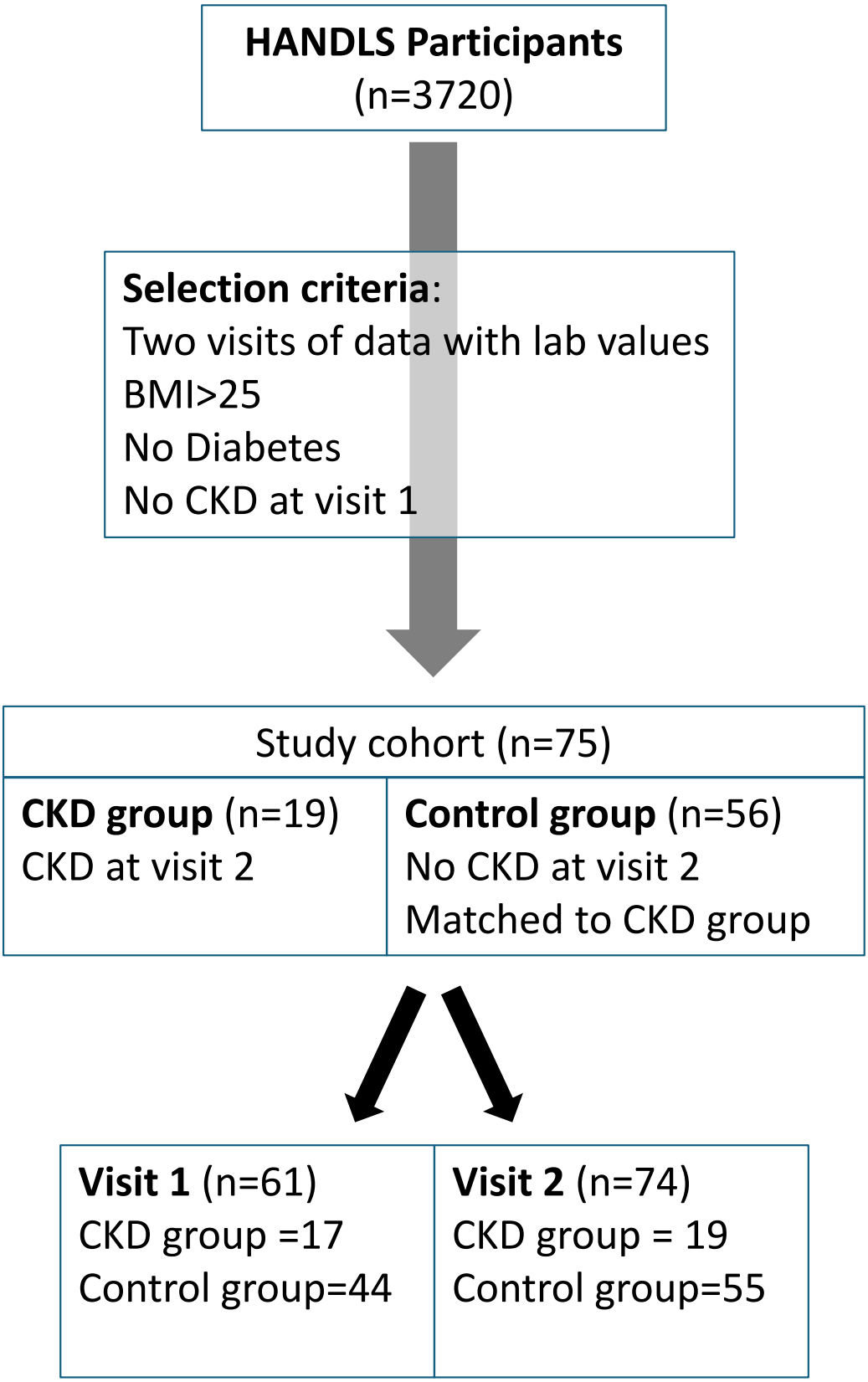
Supporting Information

Additional supporting information can be found online in the Supporting Information section.

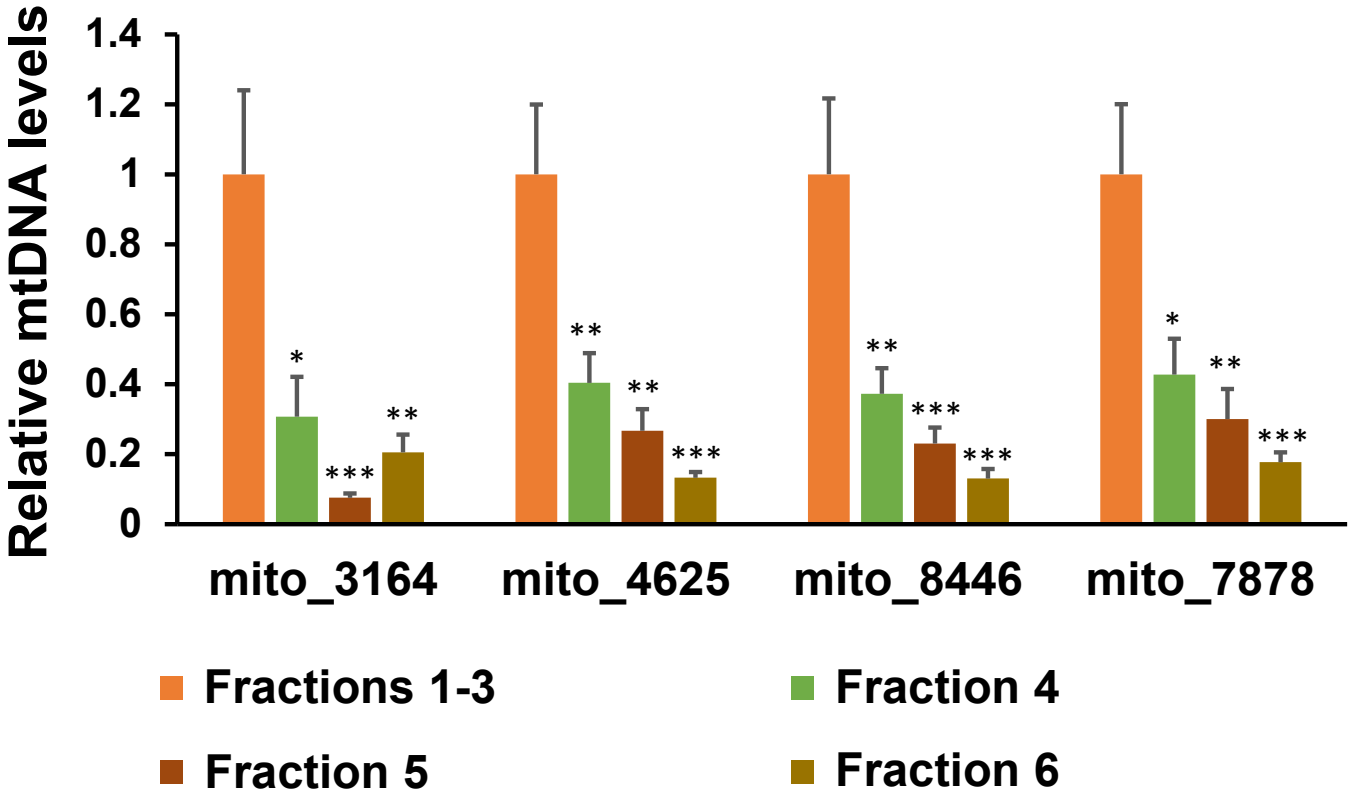
Supplemental Figure S1. Flow diagram of cohort design. Supplemental Figure S2. mtDNA levels in SEC fractions. Supplemental Figure S3. Positive correlation between EV mtDNA levels. Supplemental Figure S4. Positive correlation between EV and plasma mtDNA levels. Supplemental Table S1. Cross-sectional and longitudinal linear model results for extracellular vesicle mitochondria DNA regions by race, sex, chronic kidney disease status, poverty status, and age. Supplemental Table S2. Cross-sectional and longitudinal linear model results for plasma mitochondria DNA regions by race, sex, chronic kidney disease status, poverty status, and age Supplemental Methods. MIBlood-EV reporting tool

Supplemental Figure S1



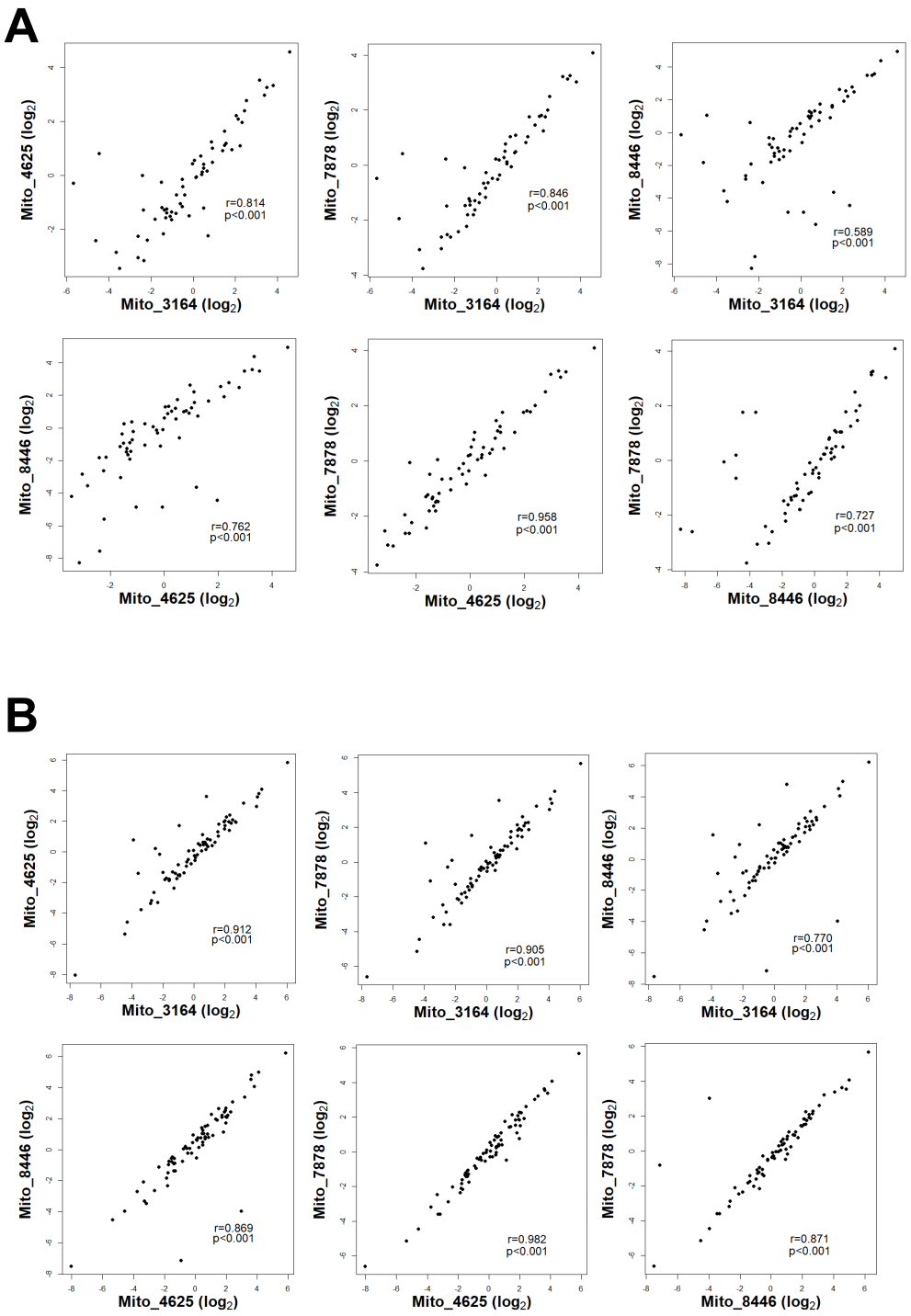
Supplemental Figure S1. Flow diagram of cohort design.

Supplemental Figure S2



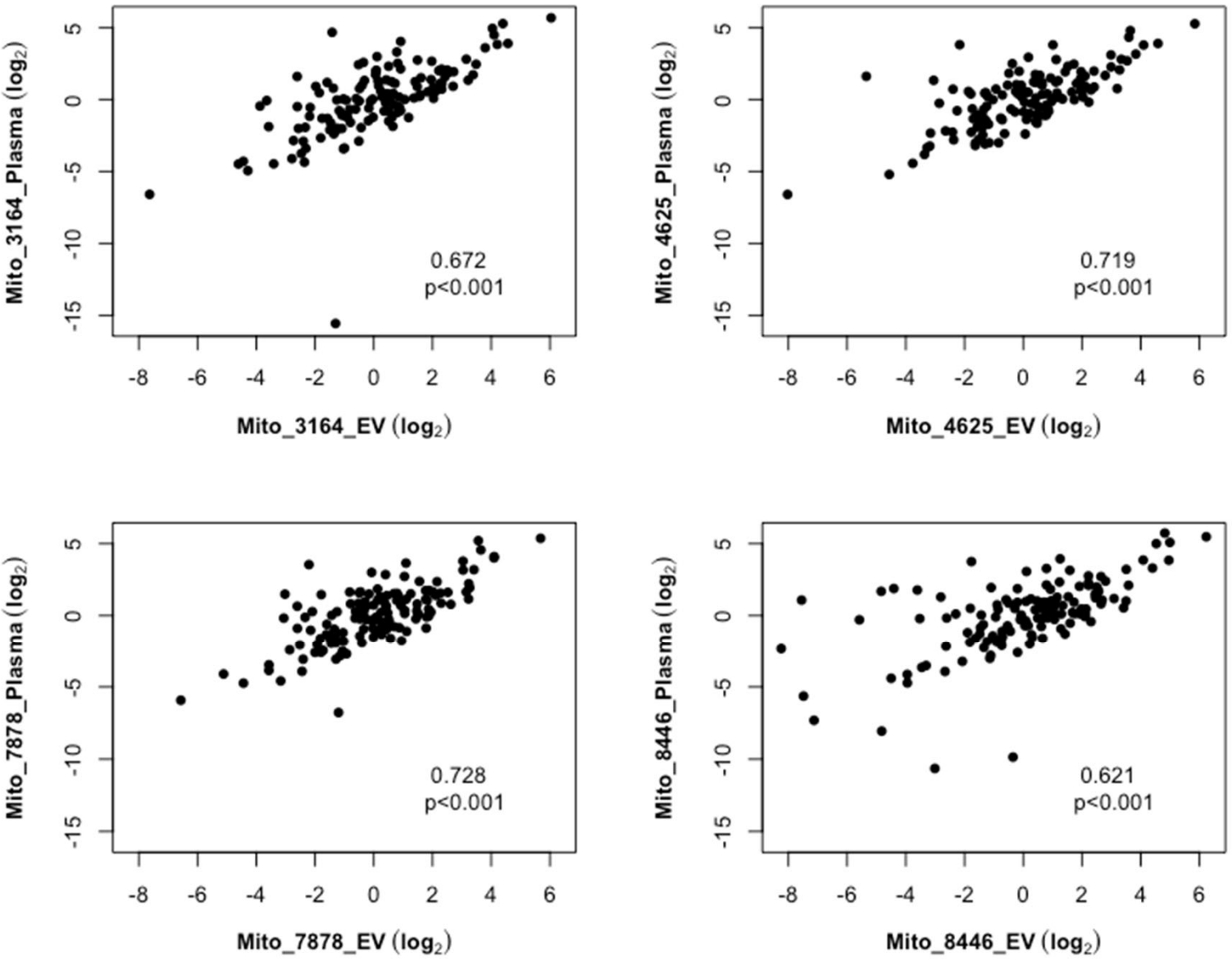
Supplemental Figure S2. mtDNA levels in SEC fractions. Plasma was pooled from 4 different individuals and plasma (500 μl) was fractionated into 10 fractions using size exclusion chromatography (SEC). Fractions 1-3 were combined for the EV-enriched fraction. DNA was isolated from equal parts of indicated SEC fractions and EV mtDNA levels were quantified using qPCR with four different mitochondrial specific primers. The histograms represents the mean from four different SEC runs with pooled plasma ± SEM. *p < 0.05, **p < 0.01 and ***p < 0.001 by Student *t* test

Supplemental Figure S3



Supplemental Figure S3. Positive correlation between EV mtDNA levels. Plasma EVs were isolated from the CKD cohort at visit 1 (A) and visit 2 (B). DNA was isolated from EVs and EV mtDNA levels were quantified using qPCR with four different mitochondrial specific primers. Each dot represents the EV mtDNA level of a participant. EV mtDNA levels (log2 transformed) were analyzed using Pearson correlation. r and p values are indicated.

Supplemental Figure S4



Supplemental Figure S4. Positive correlation between EV and plasma mtDNA levels. DNA was isolated from EVs and plasma from both visit 1 and visit 2. mtDNA levels were quantified using qPCR with four different mitochondrial specific primers. Plasma and EV mtDNA levels were log2 transformed and were analyzed using Pearson correlation. r and p values are indicated.

Supplemental Table S1. Cross-sectional and longitudinal linear model results for extracellular vesicle mitochondria DNA regions by race, sex, chronic kidney disease status, poverty status, and age

Visit 1	Race	Sex	CKD	Poverty Status	Age	Race:CKD	Poverty Status:CKD	Sex:CKD
m3164	0.972	0.376	0.341	0.151	0.617	**0.020**	**0.019**	-
m4625	0.311	0.414	0.158	0.424	0.264	-	-	-
m7878	0.243	0.366	0.289	0.447	0.180	-	-	-
m8446	0.140	0.648	0.504	0.243	0.132	-	-	**0.041**

Visit 1	Haplogroup	Sex	CKD	Poverty Status	Age	Haplogroup: CKD	Poverty Status:CKD	Sex:CKD
m3164	0.421	0.253	0.698	0.071	0.887	**0.008**	**0.019**	-
m4625	0.339	0.177	0.274	0.134	0.603	**0.024**	-	-
m7878	0.422	0.185	0.211	0.176	0.439	**0.023**	-	-
m8446	0.457	0.086	0.033	0.151	0.206	-	-	-

Longitudinal	Race	Sex	CKD	Poverty Status	Age
m3164	0.975	0.613	0.657	0.378	0.516
m4625	0.684	0.998	0.723	0.824	0.919
m7878	0.680	0.892	0.783	0.809	0.785
m8446	0.448	0.494	**0.011**	0.335	0.091

Longitudinal	Haplogroup	Sex	CKD	Poverty Status	Age
m3164	0.997	0.707	0.856	0.472	0.446
m4625	0.659	0.811	0.998	0.654	0.807
m7878	0.652	0.923	0.992	0.654	0.872
m8446	0.572	0.425	**0.021**	0.270	0.853
Stratified					
m8446	African	0.592	**0.014**	0.580	**0.023**
m8446	European	**0.027**	0.961	0.287	0.911

Longitudinal models used mixed effects regression to account for repeated measures. Interactions between two variables are indicated by a colon. P-values are presented for each coefficient and those <0.050 are in bold. Models were determined using backwards elimination from all two-way interactions and non-significant interactions were removed. Due to the correlation between race and ancestry as determined by haplogroup, only one of these terms could be in any given model. Reference values are: Race='White', Haplogroup='European', Sex='Women', CKD='Control', Poverty Status='Above'.

Supplemental Table S2. Cross-sectional and longitudinal linear model results for plasma mitochondria DNA regions by race, sex, chronic kidney disease status, poverty status, and age

Visit 1	Race	Sex	CKD	Poverty Status	Age	Race:CKD	Poverty Status:CKD	Sex:CKD	CKD:Age	Race:Age
m3164	0.817	0.840	0.693	0.393	0.820	-	-	-	-	-
m4625	0.055	0.182	0.398	0.085	0.529	**0.004**	**0.029**	-	**0.039**	-
m7878	0.869	0.439	0.656	0.804	0.357	-	-	-	-	-
m8446	0.145	0.288	0.655	0.317	0.847	**<0.001**	-	-	**0.003**	-

Visit 1	Haplogroup	Sex	CKD	Poverty Status	Age	Haplogroup: CKD	Poverty Status:CKD	Sex:CKD	CKD:Age	Race:Age
m3164	0.876	0.944	0.954	0.517	0.808	-	-	-	-	-
m4625	0.086	0.110	0.044	0.257	0.906	**0.005**	-	-	-	-
m7878	0.087	0.197	0.040	0.365	0.933	**0.008**	-	-	-	-
m8446	0.121	0.224	0.427	0.288	0.934	**<0.001**	-	-	**0.002**	-

Longitudinal	Race	Sex	CKD	Poverty Status	Age	Race:CKD	Poverty Status:CKD	Sex:CKD	CKD:Age	Race:Age
m3164	0.398	0.326	0.545	0.162	0.701	-	-	-	-	-
m4625	**0.048**	0.366	0.385	0.660	0.488	-	-	-	-	-
m7878	0.091	0.490	0.236	0.454	0.645	-	-	-	-	-
m8446	0.580	0.550	0.482	0.722	**0.007**	**<0.001**	-	-	**<0.001**	**0.006**

Longitudinal	Haplogroup	Sex	CKD	Poverty Status	Age	Haplogroup: CKD	Poverty Status:CKD	Sex:CKD	CKD:Age	Race:Age
m3164	0.556	0.376	0.498	0.188	0.617	-	-	-	-	-
m4625	0.105	0.428	0.266	0.655	0.311	-	-	-	-	-
m7878	0.153	0.589	0.178	0.498	0.474	-	-	-	-	-
m8446	**0.044**	0.888	0.054	0.791	**0.327**	**0.001**	-	-	**<0.001**	-

Longitudinal models used mixed effects regression to account for repeated measures. Interactions between two variables are indicated by a colon. P-values are presented for each coefficient and those <0.050 are in bold. Models were determined using backwards elimination from all two-way interactions and non-significant interactions were removed. Due to the correlation between race and ancestry as determined by haplogroup, only one of these terms could be in any given model. Reference values are: Race='White', Haplogroup='European', Sex='Women', CKD='Control', Poverty Status='Above'.

INTERNATIONAL SOCIETY for EXTRACELLULAR VESICLES

MIBlood-EV

Standardized Reporting Tool for Blood EV Research (Human)

STUDY INFORMATION

1.0 **Manuscript title**	Extracellular vesicle mitochondrial DNA levels are associated with chronic kidney disease and mitochondrial haplogroup in obese individuals		
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**	135
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)**	2004-2014

BLOOD COLLECTION AND PROCESSING

2.0 **Patient fasting status**	Fasted	2.1 **Fasting length (e.g. hours/days)**	overnight
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)**	9-19

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**			