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Blood-derived mitochondrial DNA copy number is associated with Alzheimer disease, Alzheimer-related biomarkers and serum metabolites

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Abstract

Background Blood-derived mitochondrial DNA copy number (mtDNA-CN) is a proxy measurement of mitochondrial function in the peripheral and central systems. Abnormal mtDNA-CN not only indicates impaired mtDNA replication and transcription machinery but also dysregulated biological processes such as energy and lipid metabolism. However, the relationship between mtDNA-CN and Alzheimer disease (AD) is unclear.

Methods We performed two-sample Mendelian randomization (MR) using publicly available summary statistics from GWAS for mtDNA-CN and AD to investigate the causal relationship between mtDNA-CN and AD. We estimated mtDNA-CN using whole-genome sequence data from blood and brain samples of 13,799 individuals from the Alzheimer's Disease Sequencing Project. Linear and Cox proportional hazards models adjusting for age, sex, and study phase were used to assess the association of mtDNA-CN with AD. The association of AD biomarkers and serum metabolites with mtDNA-CN in blood was evaluated in Alzheimer's Disease Neuroimaging Initiative using linear regression. We conducted a causal mediation analysis to test the natural indirect effects of mtDNA-CN change on AD risk through the significantly associated biomarkers and metabolites.

Results MR analysis suggested a causal relationship between decreased blood-derived mtDNA-CN and increased risk of AD (OR = 0.68; $P = 0.013$). Survival analysis showed that decreased mtDNA-CN was significantly associated with higher risk of conversion from mild cognitive impairment to AD (HR = 0.80; $P = 0.002$). We also identified significant associations of mtDNA-CN with brain FDG-PET (β = 0.103; $P = 0.022$), amyloid-PET (β = 0.117; $P = 0.034$), CSF amyloid-β (Aβ) 42/40 (β=-0.124; $P = 0.017$), CSF t-Tau (β = 0.128; $P = 0.015$), p-Tau (β = 0.140; $P = 0.008$), and plasma NFL (β=-0.124; $P = 0.004$) in females. Several lipid species, amino acids, biogenic amines in serum were also significantly associated with mtDNA-CN. Causal mediation analyses showed that about a third of the effect of mtDNA-CN on AD risk was mediated by plasma NFL ($P = 0.009$), and this effect was more significant in females ($P < 0.005$).

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Conclusions Our study indicates that mtDNA-CN measured in blood is predictive of AD and is associated with AD biomarkers including plasma NFL particularly in females. Further, we illustrate that decreased mtDNA-CN possibly increases AD risk through dysregulation of mitochondrial lipid metabolism and inflammation.

Keywords Mitochondria DNA copy number, Biomarkers, Whole genome sequencing, Alzheimer's disease, Serum metabolites, Mendelian randomization, Causal mediation

Background

Mitochondria are double-membrane organelles and the site of many vital cellular processes, including the generation of ATP through oxidative phosphorylation (OXPHOS), apoptosis, lipid metabolism, and maintaining calcium homeostasis [1, 2]. Human mitochondria have ~16.6 kb-long circular DNA (mtDNA) encoding 2 rRNAs, 22 tRNAs, and 13 OXPHOS proteins. A human cell can contain 0–600,000 mitochondria and each mitochondrion contains 1–10 copies of mtDNA [3, 4]. mtDNA copy number (mtDNA-CN) is cell-type-specific and developmental-stage-specific, depending on cellular energy demand. Thus, mtDNA-CN is also a proxy measurement of mitochondrial function. Abnormal mtDNA-CN not only indicates impaired mtDNA replication and transcription machinery [5, 6], but also suggests dysregulated biological processes, leading to mitochondrial dysfunction. Low blood-derived mtDNA-CN is a risk factor for several peripheral diseases, including cardiometabolic disease, type 2 diabetes, chronic kidney disease, and dementia [5, 7–10].

Late-onset Alzheimer disease (AD) is the most common cause of dementia. It is estimated that 6.5 million people aged 65 and older have AD in the United States, and this number will increase to 13.8 million by 2060 [11]. The role of β-amyloid (Aβ) and tau proteins in AD pathogenesis have been intensively studied in the past few decades, but their underlying mechanisms remain unclear [12]. Mitochondrial dysfunction has also been extensively studied and may become a novel therapeutic target for AD [13, 14]. One feature of mitochondrial dysfunction is disturbed mitochondrial genome homeostasis, which is in part reflected by abnormal mtDNA levels [6, 13]. Decreased mtDNA-CN in pyramidal neurons from AD cases compared to cognitively normal (CN) individuals has been observed [15]. Similarly, lower mtDNA-CN was found in peripheral mononuclear blood cells from AD and mild cognitive impairment (MCI) patients compared to CN subjects [16]. These findings suggest that mtDNA-CN may be a marker of mitochondrial dysfunction in a preclinical stage of AD. However, the metabolic mediators of mitochondria and mechanistic connection between blood mtDNA-CN and AD remain unknown. Yang et al. observed that genes whose expression was significantly associated with blood mtDNA-CN were enriched in neurodegenerative disease pathways in multiple tissues [5]. A direct connection of mtDNA-CN to AD was established by a genome-wide association study (GWAS) of blood mtDNA-CN which identified significant associations with genes involved in Aβ clearance [17].

In this study, we employed multiple analytical approaches to evaluate the association of AD with mtDNA-CN estimated from whole genome sequence (WGS) data from more than 13,000 participants of the Alzheimer's Disease Sequencing Project (ADSP) and to determine the causal relationship between mtDNA-CN and AD using publicly available GWAS summary statistics. We also examined the association of mtDNA-CN with a panel of proteins and metabolites measured in the Alzheimer Disease Neuroimaging Initiative (ADNI) participants. Finally, we investigated possible mediation by proteins and metabolites of the association between mtDNA-CN and AD.

Methods

Overview of analysis design

As depicted in Fig. 1, we assessed associations of mtDNA-CN with AD and pertinent covariates including age, sex, APOE4 status, and ancestry in WGS data derived from brain and blood tissue of ADSP participants. Analyses were performed separately for each ancestry group because the heritability of mtDNA-CN varies among populations and associations of mtDNA-CN with other traits is influenced by environmental factors [18]. The effect of mtDNA-CN level on conversion of CN to MCI and MCI to AD was assessed by survival analysis. Next, we tested the hypothesis that low blood mtDNA-CN is a causal factor to AD using Mendelian Randomization (MR). We also evaluated associations of blood mtDNA-CN with AD biomarkers and metabolites in ADNI datasets using linear regression models. AD status-stratified and sex-stratified analyses were performed to investigate how these associations change across AD stages and between genders. Causal mediation analysis was also conducted to identify which biomarkers and metabolites mediate the association between blood mtDNA-CN and AD.

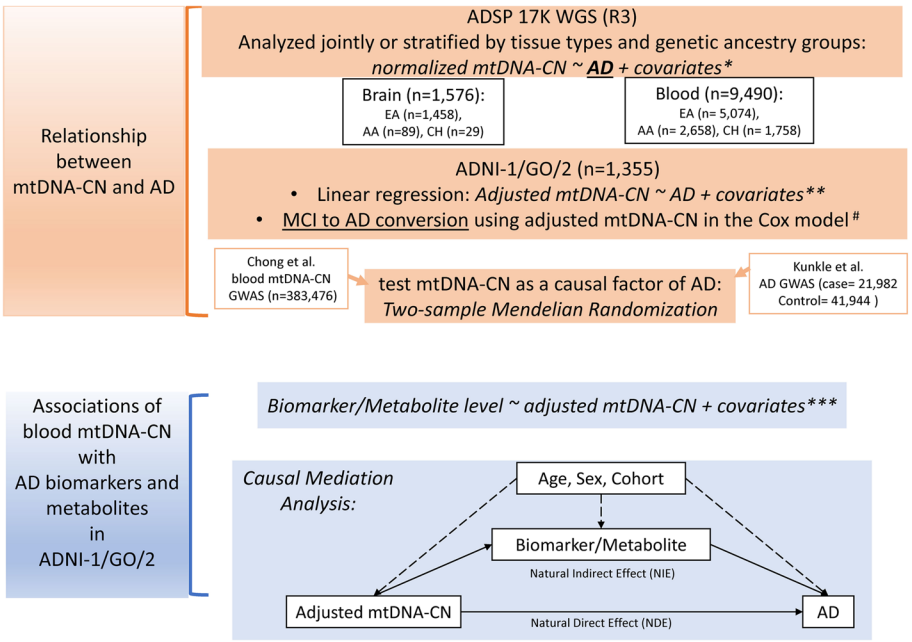

Fig. 1 Analysis design. mtDNA-CN = mitochondria DNA copy number; ADSP = Alzheimer's Disease Sequencing Project; WGS = whole genome sequencing; AD = Alzheimer disease; EA = European Ancestry, AA = African American, CH = Caribbean Hispanic; ADNI = Alzheimer's Disease Neuroimaging Initiative; MCI = mild cognitive impairment; GWAS = genome-wide association study; * Covariates include age, sex, genetic ancestry group, *APOE* ε2 and ε4 dosage, sequencing center, and PCR. ** Covariates include age, sex, and original study phase; *** Covariates include age, sex, original study phase, and AD diagnosis; # Cox proportional hazards regression model: $\log h(t) \sim adjusted\ blood\ mtDNA - CN + age + sex + original\ study\ phase$

Subjects and whole genome sequencing

Study participants were selected from cohorts assembled by the ADSP for whole genome sequencing. Details of subject ascertainment, diagnostic procedures, and WGS methods using DNA obtained from blood or brain tissue for the ADSP Release 3 (R3) dataset containing 16,774 individuals are described elsewhere [19–21]. The Genome Center for Alzheimer's Disease (GCAD) quality control (QC) working group performed quality checks of variants and genotypes and samples [19]. Individuals with unknown AD status ($n = 2{,}774$) and unexpected genetic duplicate records ($n = 286$) were excluded, yielding a sample of 13,799 individuals for subsequent analyses (Additional file 2: Table S1). Individuals in the pre-filtered R3 dataset were clustered into population ancestry groups using a Gaussian Mixture model applied to principal component analysis performed with the GENESIS R package [22]. 2,930, 3,027, and 7,842 individuals were identified to be genetically similar to African Americans (AA), Caribbean Hispanics (CH), and Europeans (EA) respectively. Some of the subjects in this dataset were members of multiplex families (comprising two or more individuals affected with AD). The R3 dataset included 1,575 participants in multiple phases of ADNI (ADNI-1, ADNI-GO, ADNI-2) which is a multicenter longitudinal study of MCI and AD that performs cognitive and neuroimaging exams and measures AD biomarkers derived from CSF and blood [23]. WGS for the ADNI samples was performed in two batches several years apart using different sequencing platforms. The distribution of ADNI subjects according to diagnosis and study phase is shown in Table 1.

Estimation of mtDNA-CN

We developed a custom pipeline to estimate mtDNA-CN from WGS data in CRAM files. Genome Analysis Toolkit version 4.0 was used to separate mtDNA read alignments from nuclear read alignments in each CRAM file (Additional file 1: Fig. S1). Paired-end reads that mapped to two different chromosomes were discarded. To efficiently estimate autosomal DNA coverage, we randomly sampled 3,000 1,000-bp regions that meet the following criteria for estimation: (1) no overlap with telomeres, centromeres, gaps, and low complexity regions, and (2) matched average GC content with mtDNA. The mitochondrial control region and both ends with an average read length of the non-control region were excluded from mtDNA coverage estimation for each sequencing library due to their low mapping quality. Mosdepth software was used to count base coverage for both autosomal DNA and mtDNA [24]. mtDNA-CN was estimated as

Table 1 Demographic characteristics of unrelated ADNI participants with European ancestry

Cohorts	ADNI-1	ADNI-GO	ADNI-2
Total, No.	585	109	661
Age, mean (SD), y	74.8 (6.8)	71.5 (7.6)	72.4 (7.1)
Female, No. (%)	233 (39.8%)	47 (43.1%)	307 (46.4%)
Baseline Diagnosis, No. (%)			
CN	161 (27.5%)	-	238 (36.0%)
MCI	301 (51.5%)	109 (100%)	300 (45.4%)
AD	123 (21.0%)	-	123 (18.6%)
Progression from CN to MCI, No. (%)			
Yes	38 (26.2%)	-	25 (11.7%)
No	107 (73.8%)	-	189 (88.3%)
Progression from MCI to AD, No. (%)			
Yes	155 (57.2%)	12 (14.6%)	77 (30.8%)
No	116 (42.8%)	70 (85.4%)	173 (69.2%)

SD Standard deviation, *CN* Cognitively normal, *MCI* Mild cognitive impairment, *AD* Alzheimer's Disease, *ADNI* the Alzheimer's Disease Neuroimaging Initiative

$$2 \times \frac{mean\ base\ coverage\ of\ mtDNA}{mean\ base\ coverage\ of\ autosomes}$$

We compared our customized pipeline with fastMitoCalc [25] by leveraging the 39 technical replicates derived from blood samples. These replicates included three subjects sequenced 9 times ($n=27$) and two subjects sequenced 6 times ($n=12$). As shown in Fig. S2 (Additional file 1), the mean mtDNA-CN that was estimated using our pipeline (mean = 220.3) was significantly less compared to the mean estimated using fastMitoCalc (mean = 231.2; ANOVA $P=9.61\times10^{-9}$).

Preprocessing/Normalization of blood mtDNA-CN and calculation of polygenic risk scores

Previous studies reported that leukocytes and platelets counts are major confounding factors in the estimation of mitochondrial genome copy number per cell [5, 17, 26, 27]. To adjust for the effect of blood cell type composition on blood mtDNA-CN level estimated in the ADNI dataset, PRSice-2 was used [28] to calculate polygenic risk scores (PRS) by leveraging summary statistics of blood cell count GWAS recently published by Chen et al. [29]. Variants with association p-values $<5\times10^{-5}$ and minor allele frequency > 0.01 from each blood cell count trait GWAS for individuals with European ancestry were selected and then LD-clumped (r2 < 0.1) for the PRS calculation. PRS of neutrophils ($P=1.53\times10^{-6}$), lymphocytes ($P=0.15$), and platelets ($P=0.007$) and a binary variable of whether the sequencing library was PCR amplified ($P=7.96\times10^{-65}$) were selected using stepwise regression in both directions and included as covariates in a linear regression model with mtDNA-CN as the outcome. Standardized and inverse normal transformed residuals from linear regression were used as adjusted mtDNA-CN for all subsequent association analyses using ADNI data.

AD biomarker, serum metabolite and plasma protein measurements

Baseline measurements of AD biomarkers including fluorodeoxyglucose (FDG) and amyloid-β (Aβ) measured by positron emission tomography (PET) scan and plasma and CSF measures of Aβ42, Aβ40, total tau (t-tau), phosphorylated-tau$_{181}$ (p-tau), and neurofilament light (NFL) for ADNI participants were downloaded from the Laboratory of Neuro Imaging (LONI) website (https://ida.loni.usc.edu). Measurement protocols are documented in the LONI website and were previously described [30, 31]. Data for a panel of 187 metabolites consisting of lipids, amino acids, and biogenic amines were quantified in serum samples collected from ADNI-1/GO/2 participants at the baseline visit by the Alzheimer's Disease Metabolomics Consortium (ADMC) [32], as well as for another panel of 190 proteins that were measured in plasma samples collected at baseline from ADNI-1 participants by the Biomarkers Consortium Plasma Proteomics Project were downloaded from the LONI website.

Statistical analysis methods

Two-sample mendelian randomization analysis

To test the hypothesis that lower mtDNA-CN in blood is causally related to increased risk of AD, we performed two-sample MR analyses using summary statistics from two independent GWAS because data for multiple traits were unavailable for the same cohort. We selected 26 single nucleotide polymorphisms (SNPs) as instrument variables from a blood mtDNA-CN GWAS focused on mitochondria disorder-related traits [10]. Summarized results were also obtained for 24 of these SNPs from a large AD GWAS [33]. Data from the two GWAS were harmonized to align the effect allele for each SNP. A synonymous variant, rs62641680, in the *DGUOK* loci was found to largely influence causal effect estimate in the leave-one-out analysis using MR-Egger (Additional file 2: Table S2) and therefore was excluded, which resulted in 23 SNPs in the final analysis. MR analysis was performed using several approaches including inverse variance weighted (IVW), median weighted, MR-Egger, and robust adjusted profile score (RAPS), which models pleiotropy to overcome the bias caused by violation of the exclusion restriction assumption [34–37], that are implemented in the R package "TwoSampleMR" (version 0.5.6) [38]. MR-PRESSO and HEIDI methods, implemented in

R packages "MRPRESSO" and "gsmr" respectively, were used to detect global heterogeneity and pleiotropic outliers [39, 40]. The intercept term of MR-Egger regression was used to determine directional horizontal pleiotropy. The MR Steiger method in "TwoSampleMR" package was used to evaluate the directionality of causality [41].

Association analyses

We evaluated the association of inverse-normal-transformed mtDNA-CN with age, sex, AD status, genetic ancestry, and *APOE* ε2 and ε4 dosage, using a linear regression model adjusting for sequencing centers and a binary term of whether the sequencing library was PCR-amplified. Analyses included only unrelated individuals and were performed separately for mtDNA-CN derived from brain and blood. Additional models were tested for mtDNA-CN measured in blood for each ancestry group. A sensitivity analysis including terms for AD diagnosis and covariates measured at baseline in ADNI participants was also performed to overcome the confounding effects from time interval between age when the blood sample was drawn for WGS and age at AD diagnosis and blood cells composition. The effect of blood-derived mtDNA-CN on conversion from CN to MCI and conversion from MCI to AD in the ADNI cohort was assessed using a Cox proportional hazards model including covariates for age at baseline, sex, and study phase implemented in the R package "survival" (version 3.3-1, https://cran.r-project.org/web/packages/survival/index.html). Comparison of the rates of conversion from CN to MCI and from MCI to AD between low and high mtDNA-CN levels defined by the median of adjusted mtDNA-CN was evaluated by Kaplan-Meier survival analysis using R package "survminer" (version 0.4.9). We investigated the associations between mtDNA-CN and AD brain imaging markers measured by PET and established AD biomarkers measured in CSF and plasma. Associations of inverse normal-transformed biomarkers, metabolites, and proteins levels with the adjusted mtDNA-CN was evaluated using multiple linear regression models including covariates for age at baseline, sex, original ADNI study phase, and diagnosis at baseline. Analyses were also performed in subgroups stratified by sex or diagnosis at baseline.

Causal mediation analysis

We performed causal mediation analysis to measure the potential role of biomarkers and metabolites as mediators of the association of mtDNA-CN (i.e. exposure variable) with AD or both AD and MCI considered jointly (i.e. outcome variable) using a natural effect model that included covariates for age, sex, and study phase. This approach models the unobservable counterfactual diagnostic outcome if one was exposed to a mtDNA-CN level that is different than his/her real-world measurement and enables decomposition of the total effects of mtDNA-CN on AD risk into natural direct effect (NDE) and natural indirect effect (NIE). The model is implemented as follows. Let $Y(x, M(x))$ denote the AD diagnosis outcome observed when the mtDNA-CN is at level x and biomarker/metabolite is at level $M(x)$. In this situation, NDE captures the effect of one standard deviation increase in mtDNA-CN on AD or MCI risk with covariates at baseline levels C if one intervenes to set a biomarker or metabolite to a fixed level it would have been as if mtDNA-CN level is unchanged, which can be expressed as an odds ratio using counterfactual notation as

$$OR_{NDE} = \frac{odds\{Y(x+1, M(x)) = 1|C\}}{odds\{Y(x, M(x)) = 1|C\}}.$$

By contrast, NIE captures the effect of mtDNA-CN on AD or MCI risk only due to the effect of one standard deviation increase in mtDNA-CN on the biomarker or metabolite, which can be expressed as

$$OR_{NIE} = \frac{odds\{Y(x, M(x+1)) = 1|C\}}{odds\{Y(x, M(x)) = 1|C\}}.$$

Data were analyzed using a logistic regression model including both exposure, mediator, and covariates as predictors to obtain estimates for the outcome model. Next, 10 hypothetical exposure levels were randomly drawn for each individual from a linear model for exposure, conditioned on all covariates, and were fit in the outcome model along with the baseline levels of mediator and all covariates to impute the unobserved counterfactual outcome. Finally, this ten-fold expanded dataset was fit in the natural effect model to obtain the estimates of natural direct, indirect, and total effects using the R package medflex (version 0.6-7) [42].

Results

mtDNA-CN in brain and blood are associated with AD

In the ADSP WGS sample, we observed an average mtDNA-CN of 2,152.0 ± 1249.0 in DNA derived from brain (Additional file 1: Fig. S3A). Participants diagnosed with AD had a significantly lower brain mtDNA-CN than cognitively normal controls (β = -0.189, P=0.002, Additional file 1: Fig. S3B). Brain mtDNA-CN was positively associated with age (β=0.020, $P=2.20\times10^{-8}$, Additional file 1: Fig. S3C) and *APOE* ε2 dosage (β=0.265, $P=3.88\times10^{-4}$, Additional file 1: Fig. S3F), but not with ε4 dosage (P=0.46) or sex (P=0.76, Additional file 1: Fig. S3D). EA individuals had a significantly greater brain

mtDNA-CN compared to AAs ($P=0.006$, Additional file 1: Fig. S3E).

The average mtDNA-CN is 193.0 ± 107.4 in DNA derived from blood (Additional file 1: Fig. S4A), much lower than brain mtDNA-CN. Similar to the association findings in brain samples, blood mtDNA-CN was significantly lower in AD cases than controls (β = -0.130, $P=1.33\times10^{-9}$, Additional file 1: Fig. S4B) and positively associated with *APOE* ε2 dosage (β = 0.072, $P=0.029$, Additional file 1: Fig. S4F). However, different from associations with brain mtDNA-CN, blood mtDNA-CN was negatively associated with age (β = -0.008, $P=1.81\times10^{-11}$, Additional file 1: Fig. S4C) and significantly higher in females than males (β = 0.212, $P=2.45\times10^{-24}$, Additional file 1: Fig. S4D). Additionally, AA individuals had significantly more copies of mtDNA than EAs (β = 0.170, $P=1.80\times10^{-9}$) and significantly fewer copies of mtDNA than CH individuals (β = -0.252, $P=7.77\times10^{-14}$, Additional file 1: Fig. S4E). In ancestry-specific analyses, mtDNA-CN measured in blood was associated with lower age in EA (β = -0.011, $P=4.16\times10^{-12}$) and CH (β = -0.012, $P=1.40\times10^{-4}$) individuals and was significantly higher in females than in males in all three ancestry groups (Additional file 2: Table S3). mtDNA-CN was significantly smaller in AD cases compared to controls in the EA (β = -0.211, $P=3.58\times10^{-12}$) and CH (β = -0.263, $P=1.70\times10^{-6}$) groups.

Sensitivity analyses conducted to consider confounding effects from blood cells composition and WGS technical differences revealed that AD cases had significantly lower mtDNA-CN than MCI cases (β = -0.202, $P=0.002$) and controls (β = -0.257, $P=2.72\times10^{-4}$). However, mtDNA-CN was not different between MCI cases and controls (Fig. 2A). Lower blood mtDNA-CN was inversely associated with age (β = -0.018, $P=1.34\times10^{-7}$) (Fig. 2B) and mtDNA-CN level was higher in females than males (β = 0.426, $P=2.04\times10^{-18}$) (Fig. 2C). These results are consistent with findings from our analysis of all blood samples (Additional file 1: Fig. S4) and other studies [43].

Blood mtDNA-CN is associated with conversion from MCI to AD

Among 1,355 genetically unrelated ADNI participants of European ancestry, 63 CN individuals and 244 MCI cases converted to MCI and AD, respectively, during the follow-up period. The mean follow-up time was 5.2 years among those who converted from CN to MCI, and 3.6 years for those who converted from MCI to AD. We found that lower blood mtDNA-CN estimated at baseline visit was significantly associated with a higher risk of conversion from MCI to AD (HR = 0.80, 95% CI = 0.69–0.92, $P=0.002$). However, mtDNA-CN was not associated with conversion from CN to MCI. These observations are consistent with findings from survival analysis showing a higher probability of MCI cases with low mtDNA-CN to develop AD ($P<0.0001$; Fig. 2D), but no effect of mtDNA-CN on the conversion from CN to MCI ($P=0.28$; Fig. 2E).

Decreased blood mtDNA-CN is genetically causal to higher AD risk

After initial analyses using four two-sample MR methods, a synonymous variant, rs62641680, in the *DGUOK* loci was found to largely influence causal effect estimate in the leave-one-out analysis (Additional file 2: Table S2) and therefore was excluded, which resulted in 23 SNPs in the final analysis. Results obtained using three of the four methods indicate that decreased blood mtDNA-CN is causal to higher AD risk (IVW: β= -0.356, $P=0.044$; MR-Egger: β = -0.738, $P=0.041$; RAPS: β = -0.390, $P=0.012$; Table 2). There was no evidence of heterogeneity assessed using Cochran's Q test for MR-Egger (Q = 28.1, $P=0.14$) or IVW (Q = 30.4, $P=0.11$), additional outliers, global horizontal pleiotropy ($P=0.13$), or directional horizontal pleiotropy assessed using intercept of MR-Egger regression ($P=0.20$). The MR Steiger test of directionality indicated a valid causal direction from mtDNA-CN to AD ($P=1.93\times10^{-45}$).

Blood mtDNA-CN is associated with AD biomarkers in females and serum metabolites

In the total ADNI dataset, mtDNA-CN was nominally associated with CSF Aβ42 level ($P=0.048$), but not with any other AD biomarkers (Fig. 3, Additional file 2: Table S4). Amyloid-PET level was positively associated with mtDNA-CN in controls only (β = 0.199, $P=0.002$). mtDNA-CN was inversely associated with lower CSF Aβ42 in individuals with MCI (β = -0.102, $P=0.038$) and with higher CSF NFL level in AD cases (β = 0.281, $P=0.015$). Among females, mtDNA-CN was associated with FDG-PET, amyloid-PET, CSF t-tau and p-tau, and plasma NFL levels, whereas no significant associations were observed in males. In the total sample, mtDNA-CN was significantly associated ($_{Padj}$ < 0.05) with 26 lipid, amino acid, and biogenic amine species (Fig. 4, Additional file 2: Table S5). Significant associations were found for 14 of these metabolites, 11 of which are acylcarnitines, among individuals with MCI, but not in controls or AD cases. Among males, mtDNA-CN was significantly associated with long-chain acylcarnitines, sphingomyelins, glycine, and taurine, whereas only a negative association with hydroxyvalerylcarnitine (C5-OH, β = -0.225, $P_{adj} = 0.013$) was identified in females. Analyses of plasma protein levels revealed negative associations

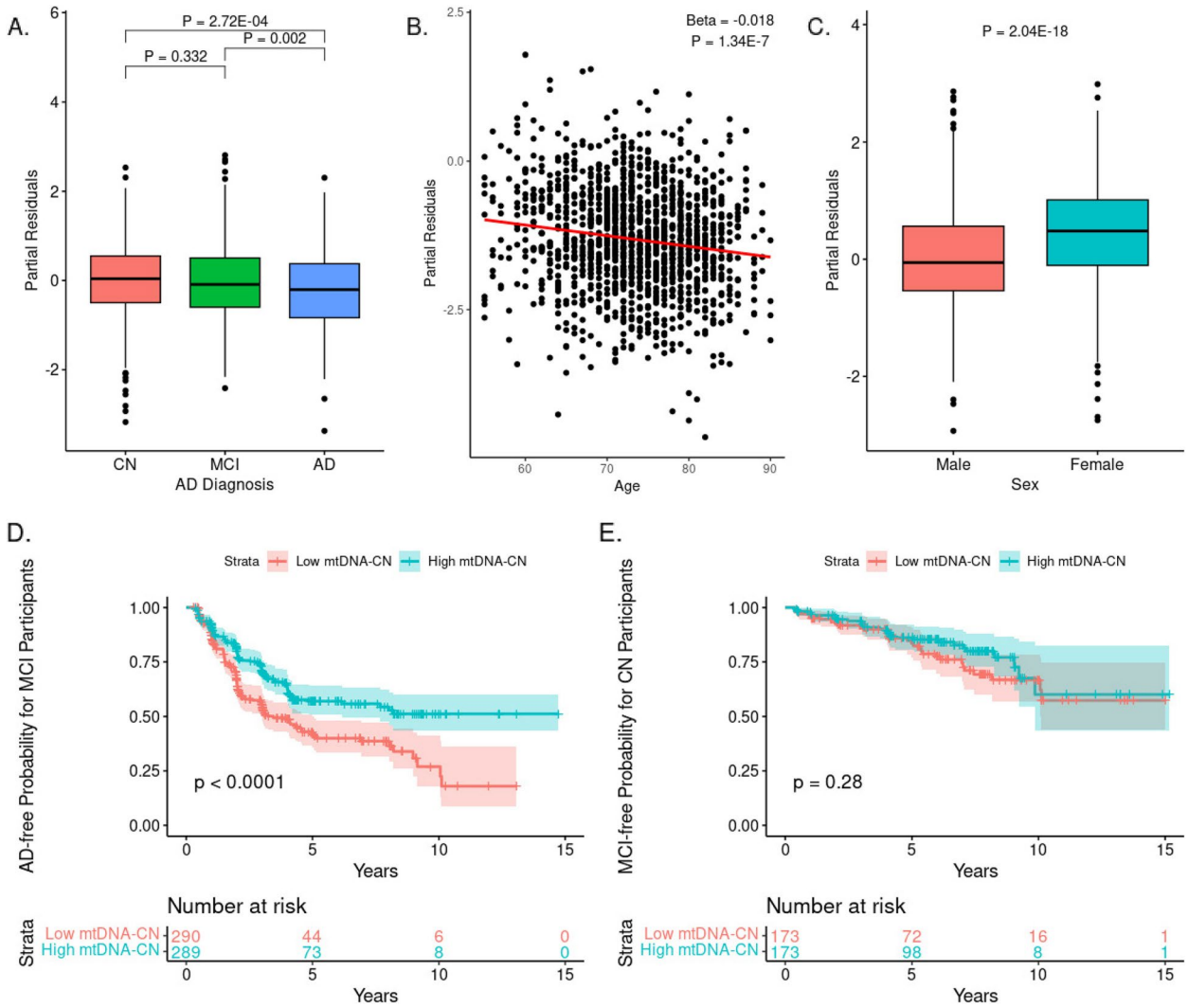

Fig. 2 Partial residuals plots showing the relationships between blood mtDNA-CN from ADNI and AD diagnosis (**A**), age (**B**), sex (**C**). Residuals were calculated after fitting a liner model where mtDNA-CN was the dependent variable and age, sex, cohort, and AD diagnosis were the independent variables. Kaplan-Meier plot to visualize the probability of (**D**) MCI participants at ADNI baseline free from converting to AD and (**E**) CN patients free from converting to MCI for low mtDNA-CN group and high mtDNA-CN group. mtDNA-CN was dichotomized by the median

Table 2 Estimation of causal effect of blood mtDNA-CN on AD using two-sample mendelian randomization methods

Method	OR	95% CI	*P*-value
Robust adjusted profile score	0.677	0.498–0.920	**0.013**
MR Egger	0.478	0.246–0.928	**0.041**
Weighted median	0.746	0.494–1.125	0.162
Inverse variance weighted	0.700	0.495–0.991	**0.044**

SE Standard error, *95% CI* 95% confidence interval

with matrix metalloproteinase 10 (MMP10) in MCI cases (β = -0.170, $P=0.044$) and matrix metalloproteinase 9 (MMP9) in males (β = -0.201, $P=0.009$) (Additional file 2: Table S6).

AD biomarkers and metabolites mediate the association of blood mtDNA-CN with AD

We found a significant natural indirect effect (NIE) of mtDNA-CN on risk of MCI or AD mediated by FDG-PET, amyloid-PET, and plasma NFL ($P<0.05$ for all). However, among these three biomarkers, a significant total effect of mtDNA-CN was observed only for plasma NFL ($P=0.039$). A natural direct effect (NDE) of mtDNA-CN on risk of MCI or AD was not evident for any mediators. In a model including only CN and AD participants, we observed very similar patterns of NIE of mtDNA-CN on AD risk mediated by FDG-PET and amyloid-PET, but the significance of NIE was attenuated. On the contrary, the NIE mediated through

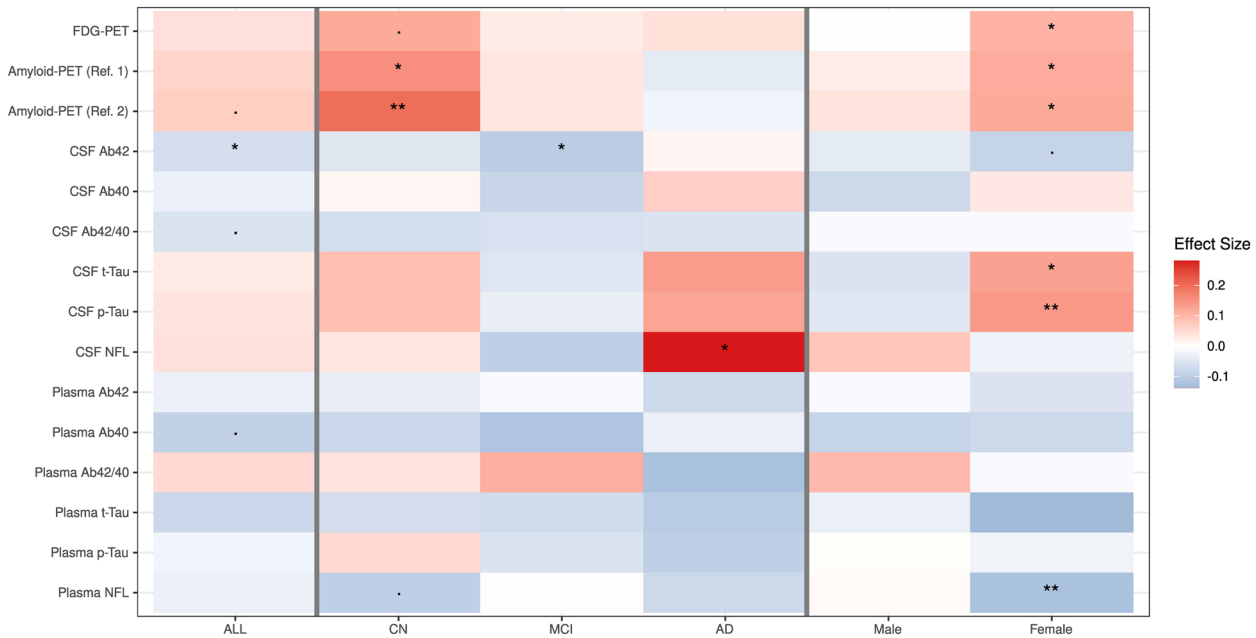

Fig. 3 *P*-value and effect size plot to visualize the association between blood mtDNA-CN and classic AD biomarkers for all ADNI participants, each sex and AD diagnosis strata. * $P < 0.05$; ** $P < 0.01$. CN = cognitively normal; MCI = mild cognitive impairment; AD = Alzheimer disease

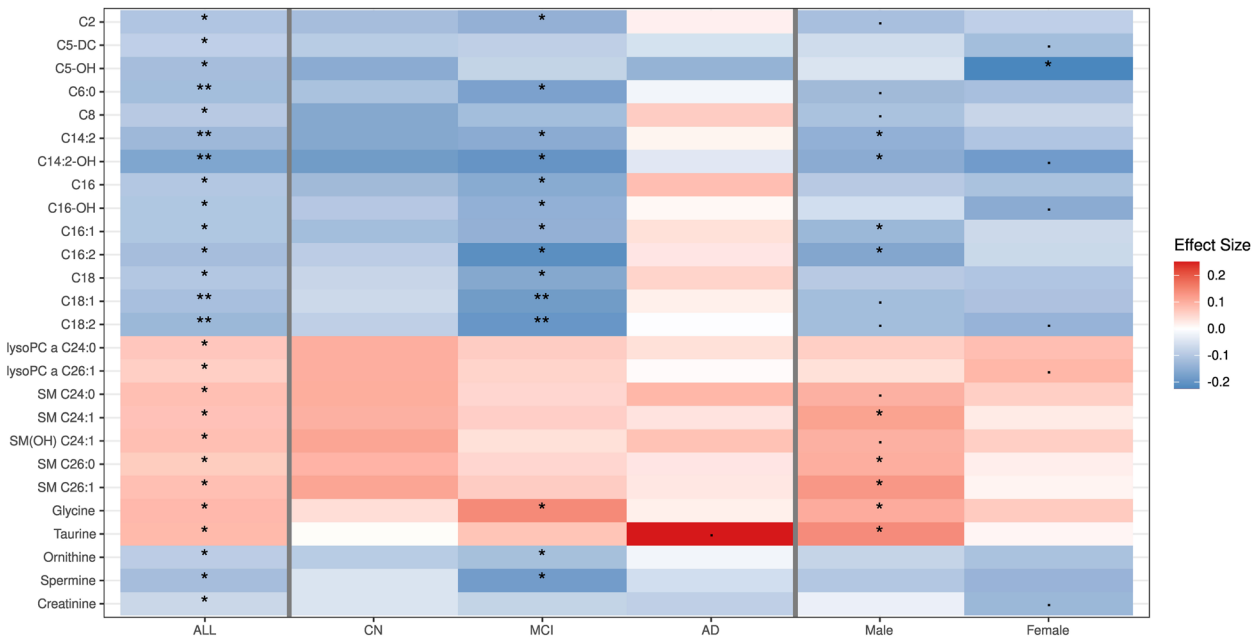

Fig. 4 Adjusted *P*-value and effect size plot to visualize the association between blood mtDNA-CN and serum metabolites for all ADNI participants, each sex and AD diagnosis strata. $P_{adj} < 0.1$; * $P_{adj} < 0.05$; ** $P_{adj} < 0.01$

plasma NFL became more significant (OR = 0.89, 95% CI = 0.82–0.96, $P = 0.002$). No significant indirect effect of mtDNA-CN was observed in a model including only MCI and AD participants in the analysis. The same results were also observed in the female subgroup, but the NIEs was larger than the total sample analysis (Table 3, Additional file 2: Table S7).

Among serum metabolites, we identified nominal NIE of mtDNA-CN on risk of MCI or AD mediated

Table 3 Mediating effects of AD biomarkers and metabolites on the association of mtDNA-CN with AD. *(To be placed at line 374, page 18)*

			NDE		NIE		TE	
Group	**Mediator**	**N**	**OR (95% CI)**	**P-value**	**OR (95% CI)**	***P*-value**	**OR (95% CI)**	***P*-value**
(MCI + AD) vs CN								
Total	**Plasma NFL**	1157	0.901 (0.784, 1.039)	0.145	0.950 (0.914, 0.987)	**0.009**	0.856 (0.740, 0.994)	0.039
	Hydroxyvalerylcarnitine	963	0.969 (0.824, 1.137)	0.698	0.950 (0.917, 0.985)	**0.005**	0.920 (0.785, 1.079)	0.306
	Octadecadienylcarnitine	1313	0.875 (0.753, 1.018)	0.082	0.980 (0.960, 0.999)	**0.044**	0.857 (0.738, 0.996)	0.044
	Glycine	1313	0.841 (0.728, 0.966)	0.017	1.018 (1.001, 1.037)	**0.042**	0.857 (0.741, 0.984)	0.033
	Serotonin	1268	0.874 (0.756, 1.009)	0.066	0.983 (0.965, 1.002)	0.073	0.859 (0.743, 0.993)	0.040
Male	Plasma NFL	642	0.860 (0.705, 1.047)	0.135	0.980 (0.934, 1.029)	0.411	0.843 (0.689, 1.031)	0.097
	Hydroxyvalerylcarnitine	746	0.881 (0.725, 1.072)	0.204	0.967 (0.934, 1.001)	0.054	0.852 (0.699, 1.038)	0.111
	Octadecadienylcarnitine	746	0.881 (0.717, 1.080)	0.225	0.967 (0.935, 0.999)	**0.045**	0.852 (0.693, 1.045)	0.125
	Glycine	746	0.824 (0.681, 1.005)	0.051	1.032 (1.001, 1.065)	**0.042**	0.850 (0.704, 1.037)	0.099
	Serotonin	722	0.882 (0.731, 1.064)	0.189	0.991 (0.969, 1.015)	0.456	0.874 (0.723, 1.056)	0.163
Female	**Plasma NFL**	515	0.954 (0.772, 1.183)	0.666	0.919 (0.868, 0.977)	**0.005**	0.877 (0.705, 1.099)	0.246
	Hydroxyvalerylcarnitine	410	1.076 (0.848, 1.367)	0.547	0.897 (0.830, 0.970)	**0.007**	0.966 (0.760, 1.229)	0.775
	Octadecadienylcarnitine	567	0.871 (0.703, 1.082)	0.211	0.997 (0.970, 1.028)	0.859	0.869 (0.702, 1.076)	0.198
	Glycine	567	0.862 (0.694, 1.067)	0.176	1.008 (0.985, 1.031)	0.476	0.869 (0.700, 1.079)	0.205
	Serotonin	546	0.864 (0.700, 1.091)	0.198	0.979 (0.949, 1.009)	0.165	0.846 (0.676, 1.057)	0.141
AD vs CN								
Total	**Plasma NFL**	609	0.848 (0.715, 1.008)	0.059	0.886 (0.822, 0.960)	**0.002**	0.751 (0.625, 0.911)	0.003
	Hydroxyvalerylcarnitine	457	0.855 (0.683, 1.076)	0.177	0.975 (0.942, 1.007)	0.135	0.834 (0.666, 1.046)	0.114
	Octadecadienylcarnitine	625	0.722 (0.595, 0.871)	0.001	0.992 (0.976, 1.010)	0.373	0.716 (0.591, 0.866)	0.001
	Glycine	625	0.713 (0.597, 0.862)	3.25×10^{-4}	1.004 (0.982, 1.026)	0.709	0.716 (0.598, 0.866)	4.16×10^{-4}
	Serotonin	611	0.744 (0.617, 0.905)	0.002	0.970 (0.941, 1.000)	**0.048**	0.722 (0.599, 0.879)	0.001
Male	Plasma NFL	322	0.826 (0.662, 1.043)	0.099	0.958 (0.860, 1.065)	0.431	0.791 (0.613, 1.021)	0.072
	Hydroxyvalerylcarnitine	331	0.767 (0.602, 0.996)	0.039	0.986 (0.954, 1.018)	0.393	0.756 (0.587, 0.974)	0.030
	Octadecadienylcarnitine	331	0.767 (0.598, 0.983)	0.037	0.986 (0.955, 1.018)	0.382	0.756 (0.589, 0.970)	0.028
	Glycine	331	0.751 (0.590, 0.965)	0.022	1.009 (0.974, 1.047)	0.623	0.757 (0.593, 0.979)	0.030
	Serotonin	325	0.802 (0.625, 1.046)	0.094	0.979 (0.944, 1.016)	0.255	0.786 (0.607, 1.017)	0.067
Female	**Plasma NFL**	287	0.882 (0.676, 1.139)	0.344	**0.813** (0.724, 0.920)	**0.001**	0.717 (0.540, 0.950)	0.021
	Hydroxyvalerylcarnitine	211	0.869 (0.607, 1.240)	0.440	0.936 (0.861, 1.024)	0.135	0.813 (0.573, 1.158)	0.249
	Octadecadienylcarnitine	294	0.680 (0.504, 0.925)	0.013	0.998 (0.979, 1.019)	0.818	0.679 (0.502, 0.917)	0.012
	Glycine	294	0.678 (0.513, 0.910)	0.008	1.001 (0.968, 1.037)	0.945	0.679 (0.510, 0.903)	0.008
	Serotonin	286	0.683 (0.508, 0.935)	0.015	0.964 (0.913, 1.016)	0.173	0.658 (0.485, 0.893)	0.007

NIE Natural indirect effect, *NDE* Natural direct effect, *TE* Total effect, *CN* Cognitively normal, *MCI* Mild cognitive impairment, *AD* Alzheimer disease, *OR* Odds ratio, *CI* Confidence interval, *NFL* Neurofilament light

by two acylcarnitine species, C5-OH (OR = 0.95, 95% CI = 0.92–0.99, $P = 0.005$) and octadecadienylcarnitine (C18:2: OR = 0.98, 95% CI = 0.96-1.00, $P = 0.044$), and glycine (OR = 1.02, 95% CI = 1.00-1.04, $P = 0.042$). The NIE of mtDNA-CN was mediated by serotonin (OR = 0.97; 95% CI = 0.94-1.00, $P = 0.048$) in a model including only CN and AD participants. Among females, the NIE of mtDNA-CN on risk of MCI or AD was mediated only by C5-OH ($P = 0.007$), whereas nominally significant NIE through C18:2 ($P = 0.045$) and glycine ($P = 0.042$) was observed in males (Table 3, Additional file 2: Table S8).

Discussion

This study leveraged whole genome sequence data obtained from a large sample of ADSP participants to measure mtDNA-CN that is relatively difficult to quantify by traditional genotyping methods [44, 45]. We observed that participants with AD diagnoses had lower mtDNA-CN levels in both brain and blood samples compared to cognitively normal individuals, which is consistent with

previous findings [46, 47]. This finding was evident in the EA and CH groups, but not in AAs. MR analysis results suggest that decreased blood mtDNA-CN is causal to AD (OR = 0.68; $P = 0.012$). We also identified associations of AD biomarkers and metabolites with mtDNA-CN some of which showed sex- and AD status-specific patterns. Our study further characterized the mediators in the association of mtDNA-CN with AD/MCI, indicating the dysfunction of mitochondria for the disease.

In the total sample, mtDNA-CN was positively associated with the *APOE* ε2 allele in both brain and blood samples. Longchamps et al. also identified a positive association between blood-derived mtDNA-CN and the ε2 SNP (rs7412) [17]. This finding suggests a protective effect of *APOE* ε2 against mitochondrial dysfunction. Although we did not observe a significant association of mtDNA-CN with the *APOE* ε4 allele, another study of AD cases reported evidence of impaired mitochondria and oxidative stress in brain tissue from ε4 carriers [48, 49].

We identified female-specific associations of blood mtDNA-CN with several AD biomarkers, including FDG-PET, amyloid-PET, CSF Aβ42/40, CSF t-Tau, CSF p-Tau, and plasma NFL. Previous studies reported sex differences in the association of AD with these biomarkers, except plasma NFL, and related metabolic pathways [50–53], suggesting that unidentified sex-specific factors modify the effect of mtDNA-CN on AD-related processes. However, in the causal mediation analysis, the natural indirect effect and total effect of mtDNA-CN on AD were simultaneously significant when testing for plasma NFL, but not the other AD biomarkers, as a mediator. Plasma NFL has been proposed as a prognostic biomarker for AD because of its association with AD risk, cognitive decline, and chronic inflammation [54–56]. It is shown that immune dysfunction, energy demands, inflammation and altered cell signaling link mitochondrial dysfunction in whole blood to chronic diseases especially cardiovascular diseases [57]. In addition, previous study of ADNI participants showed higher plasma NFL was predictive of brain glucose hypometabolism, which is associated with mitochondrial dysfunction [58], in cognitively impaired and normal subjects [59]. These observations combined with our findings suggest low numbers of mitochondria probably do not respond well to the stressors of inflammation and vascular diseases, leading to inefficient oxidative phosphorylation in mitochondria and AD pathogenesis.

The association findings for mtDNA-CN with amyloid are seemingly inconsistent. Whereas mtDNA-CN was nominally associated with lower CSF Aβ42 in the total sample, it was more significantly associated with increased amyloid-PET level in controls, but not in MCI or AD participants. The evidence for a relationship between amyloid deposition and mtDNA-CN in brain is controversial. Decreased Aβ42 level along with reduced mtDNA-CN was observed in the cortex and hippocampus of an AD transgenic mice that express mitochondrial-targeted endonuclease Mito-*PstI* [60, 61], whereas another study reported a nominally significant negative association in the dorsolateral prefrontal cortex from post-mortem normal ageing and AD brains [46]. Because blood mtDNA-CN is not necessarily a proxy for brain mtDNA-CN, further investigation is needed to fully understand the interaction between amyloid and mtDNA.

Several studies have identified associations of protein and metabolite levels with AD and cognitive decline in the ADNI cohort [32, 62, 63]. For example, it has been suggested that a combination of markers other than β-amyloid and tau measured in plasma and CSF could prove useful in predicting progression from MCI to AD [51]. Recently, Horgusluoglu et al. showed that short-chain acylcarnitines/amino acids and medium/long-chain acylcarnitines are associated with episodic memory scores and disease severity [63]. Our study, which compared mtDNA-CN with metabolites measured in the same blood sample, identified associations with several lipid species, amino acids, and biogenic amines. Most notably, lower mtDNA-CN was associated with levels of acylcarnitines (ACs). ACs have essential roles in mitochondrial metabolism because they transport fatty acids through mitochondrial membranes for β-oxidation. They shuttle across mitochondrial membranes and are recycled primarily by long-chain acyl-coenzyme A synthetase, carnitine/acylcarnitine translocase, and carnitine palmitoyl-transferase 1 and 2 under normal physiological conditions. We observed that AD cases had elevated levels of blood medium/long-chain ACs, which may be attributed to damaged mitochondria with reduced fatty acid oxidation rate [63]. Curiously, most of our observed associations of mtDNA-CN with ACs were evident primarily in MCI participants. Lack of association of mtDNA-CN with AC levels among AD cases combined with results from a previous study showing that AC levels progressively decline with transitions from cognitively normal to AD [64] suggest there is a floor effect for the impact of the interaction of blood mtDNA-CN with AC levels on AD progression when cognitively declining individuals reach the AD stage. Our findings that mtDNA-CN was associated with levels of four long-chain ACs in males and reduced level of the short-chain acylcarnitine C5-OH was associated with mtDNA-CN in females were supported by mediation analyses which showed that the indirect natural effects mediated by the C18:2 and C5-OH ACs were significant only in males and females,

respectively. Although sex differences in AC metabolism have been previously reported [65, 66], mechanisms underlying sex-specific relationships between AC levels and mtDNA-CN are unclear and require further investigation.

Although the association between blood mtDNA-CN and serotonin was not significant after FDR correction, the NIE of mtDNA-CN on AD mediated by serotonin was significant. Serotonin is a key neurotransmitter and its concentration in serum and urine is significantly lower in AD cases compared to controls. It has been suggested that the disruption of serotonergic signaling pathway is linked to enhanced amyloid pathology and, thus, serotonin may be a potential therapeutic target [67]. Because mitochondria provide ATP which is essential to serotonin signaling, mitochondrial dysfunction could result in serotonin dysregulation and subsequent neurodegeneration, an idea that is consistent with our causal mediation analysis findings [68].

Sphingomyelin (SM) is the major type of sphingolipid in blood and has important biological functions including cellular signaling and membrane stabilization. The hydrolysis of SM is the major source of ceramide, which can act as a second messenger and trigger mitochondrial apoptosis. Studies have indicated that Aβ induces apoptosis through SM/ceramide pathway and reduced SM levels have been observed in AD brains and plasma [69–71]. The positive associations we observed between a few SM species and mtDNA-CN, might suggest the degradation of SM results in mitochondrial dysfunction. In addition, there is evidence that plasma SM concentrations are higher and increase faster with age in older females than males [72]. Mielke et al. reported that high plasma SM level is associated with increased AD risk in men, but is protective against AD in women [73]. Interestingly, we observed associations of mtDNA-CN with SMs in males only, suggesting the interaction between SM and mitochondria, especially SM hydrolysis-induced apoptosis pathway, differs between genders.

We also identified associations of blood mtDNA-CN with serum levels of ornithine, spermine, creatinine, and 2 lysophosphatidylcholine (lysoPC) species in the total sample, and with glycine and taurine in males only. Although altered levels of these metabolites have been implicated in mitochondrial dysfunction [74–80], there is little evidence linking them to AD.

Our study has several limitations. First, association analyses of ADSP data included age at last exam for controls and age at symptom onset for AD cases, which are both disconnected from the age when the tissue specimens were collected and, hence, for the calculation of mtDNA-CN. Moreover, age at onset for AD cases is less than the age ascribed to mtDNA-CN derived from brain, but in most instances greater than the age ascribed to mtDNA-CN derived from blood. Second, we lacked information about and thus could not incorporate in our analyses the proportions of brain or blood cell types for all ADSP participants which is an important source of confounding. Power for detecting association of AD with mtDNA-CN was considerably lower in the CH and AA groups compared to the much larger EA group. Complete blood count information was not available for ADNI participants which made it difficult to adjust mtDNA-CN estimates for blood cell composition, which has been recognized as an important confounder in large-scale mtDNA-CN GWAS [10, 17, 81]. Blood cell type proportions can be estimated from bulk RNA-seq data using cell-type enrichment analysis [82] or a deconvolution approach [83], but these data were available for less than half of the ADNI participants. To address this problem, we leveraged ADNI WGS data to calculate a PRS for selected blood cell types as a proxy. Third, we evaluated the association of mtDNA-CN with biomarkers and metabolite levels at baseline only and thus could not assess association patterns over the course of cognitive decline leading to AD. Lastly, the interpretation of the causal mediation analysis results may be imbalanced because data for some biomarkers and metabolites were missing for varying proportions of the sample. Moreover, these analyses considered only the scenario where mtDNA-CN is the exposure and biomarkers and metabolites are mediators, but not vice versa. Elucidating the role of mitochondrial dysfunction in each metabolic pathway that is involved in AD pathogenesis will require future studies.

In conclusion, we demonstrated that lower blood mtDNA-CN estimated from WGS data is causally associated with greater AD risk. In addition, we identified association of mtDNA-CN with several AD biomarkers and serum metabolites, many of which are sex-specific. Causal mediation analyses revealed mediating effects of acylcarnitine, serotonin, and plasma NFL on the influence of blood mtDNA-CN on AD risk, suggesting mitochondrial dysfunction affects multiple metabolic pathways associated with AD and mtDNA-CN is a potential blood-based biomarker. Future analyses incorporating hematology data and longitudinal mtDNA-CN measurements may yield more robust and informative findings about the role of mitochondrial dysfunction in AD.

Abbreviations

AA	African Americans
Aβ	β-amyloid
AC	Acylcarnitine
AD	Alzheimer's disease
ADMC	Alzheimer's Disease Metabolomics Consortium
ADNI	Alzheimer's Disease Neuroimaging Initiative

ADSP R3	Alzheimer's Disease Sequencing Project Release 3
C5-OH	hydroxyvalerylcarnitine
C18:2	octadecadienylcarnitine
CH	Caribbean Hispanics
CN	Cognitively normal
CSF	Cerebrospinal fluid
EA	Europeans
FDG	Fluorodeoxyglucose
GCAD	Genome Center for Alzheimer's Disease
GWAS	Genome-wide association study
IVW	Inverse variance weighted
LD	Linkage disequilibrium
LONI	Laboratory of Neuro Imaging
MCI	Mild cognitive impairment
MR	Mendelian randomization
mtDNA-CN	mitochondrial DNA copy number
NDE	Natural direct effect
NIE	Natural indirect effect
NFL	Neurofilament light
OXPHOS	Oxidative phosphorylation
p-Tau	phosphorylated-tau$_{181}$
PET	Positron emission tomography
PRS	Polygenic risk score
QC	Quality control
RAPS	Robust adjusted profile score
SNPs	Single nucleotide polymorphisms
SM	Sphingomyelin
t-Tau	total tau
WGS	Whole-genome sequence

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13195-024-01601-w.

Additional file 1: Fig. S1 Computational pipeline and equation used for estimating mtDNA-CN from WGS data. Fig. S2 Comparison of mtDNA-CN estimated by fastMitoCalc and our pipeline for each subject with WGS technical duplicates. Fig. S3 Multiple linear regression analysis for mtDNA-CN in ADSP R3 brain samples. Fig. S4 Multiple linear regression analysis for mtDNA-CN in ADSP R3 blood samples.

Additional file 2: Table S1. Demographic characteristics of ADSP R3 WGS (17 K). Table S2. Effects of non-genetic factors on blood mtDNA-CN stratified by ancestry. Table S3. Results of single SNP Ward test and leave-one-out test for 24 SNPs from Chong et al. Table S4. Associations between blood-derived mtDNA-CN and AD biomarkers in ADNI participants. Table S5. Associations between blood-derived mtDNA-CN and serum metabolites in ADNI participants. Table S6. Associations between blood-derived mtDNA-CN and plasma proteins in ADNI participants. Table S7. AD biomarkers that mediate the association between blood-derived mtDNA-CN and AD in ADNI participants. Table S8. Serum metabolites that mediate the association between blood-derived mtDNA-CN and AD in ADNI participants.

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Authors' contributions

TT and XZ designed the framework of this study. TT, CZ, ZK, and JJF pre-processed the ADSP R3 WGS data. TT performed the analyses and prepared the tables and figures. XZ, KLL, and LAF supervised the analysis. TT, XZ, LAF, KLL, WQQ, and JLH wrote the manuscript. ERM, WSB, MAP-V, L-SW, GDS, JLH and LAF obtained funding for this study. All authors edited and approved the final manuscript.

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Data availability

Data for this study are archived and distributed by the National Institute on Aging Alzheimer's Disease Data Storage Site (NIAGADS) at the University of Pennsylvania (Accession number: NG00067), funded by the National Institute on Aging (U24-AG041689). The AD biomarkers dataset, serum metabolites dataset generated by Alzheimer's Disease Metabolomics Consortium, and plasma proteomics dataset generated by Biomarkers Consortium Plasma Proteomics Project analyzed during the current study are available in the Laboratory of Neuro Imaging (LONI) Image and Data Archive (IDA) repository on reasonable request, https://ida.loni.usc.edu.

Declarations

Ethics approval and consent to participate

This study was performed in accordance with the Declaration of Helsinki and approved by the.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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