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FEATURED ARTICLE

Association of mitochondrial variants and haplogroups identified by whole exome sequencing with Alzheimer's disease

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Abstract

Introduction: Findings regarding the association between mitochondrial DNA (mtDNA) variants and Alzheimer's disease (AD) are inconsistent.

Methods: We developed a pipeline for accurate assembly and variant calling in mitochondrial genomes embedded within whole exome sequences (WES) from 10,831 participants from the Alzheimer's Disease Sequencing Project (ADSP). Association of AD risk was evaluated with each mtDNA variant and variants located in 1158 nuclear genes related to mitochondrial function using the SCORE test. Gene-based tests were performed using SKAT-O.

Results: Analysis of 4220 mtDNA variants revealed study-wide significant association of AD with a rare *MT-ND4L* variant (rs28709356 C>T; minor allele frequency = 0.002; $P = 7.3 \times 10^{-5}$) as well as with *MT-ND4L* in a gene-based test ($P = 6.71 \times 10^{-5}$). Significant association was also observed with a MT-related nuclear gene, *TAMM41*, in a gene-based test ($P = 2.7 \times 10^{-5}$). The expression of *TAMM41* was lower in AD cases than controls ($P = .00046$) or mild cognitive impairment cases ($P = .03$).

Discussion: Significant findings in *MT-ND4L* and *TAMM41* provide evidence for a role of mitochondria in AD.

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KEYWORDS
Alzheimer's disease, genetic association, mitochondrial haplogroup, mitochondrial variant calling, whole exome sequencing

1 | INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by memory loss and dementia.[1] The common form of late-onset AD among persons ages 65 years and older has a substantial genetic component with an estimated heritability of 58% to 79%.[2] Genome-wide association studies (GWAS) of common and rare variants have identified > 40 susceptibility loci in the nuclear genome,[3–10] but a large proportion of the remaining heritability of AD is still unexplained.

Mitochondria are intracellular organelles essential for cell viability by generating energy via the oxidative phosphorylation (OXPHOS) pathway. Mitochondria contain a distinct circular haploid genome of 16,569 bases. Mitochondrial (MT) function decreases with age and its dysfunction is correlated with several age-related diseases including AD.[11] Recent genetic studies have identified association of a variant in the autosomal gene encoding a subunit of mitochondrial ATP synthase, *ATP5PD*, with risk of AD and cerebral small vessel disease.[12,13] *ATP5H*, which is embedded within a larger DNA sequence that encodes *KCTD2*, has an important function in mitochondrial energy production and neuronal hyperpolarization during cellular stress conditions, such as hypoxia or glucose deprivation. However, associations of AD with mtDNA variants are inconsistent, due in part to the limited number of mtDNA variants included in genotyping arrays and lack of systematic variant calling and analysis pipelines.[14] A recent study of MT haploid genomes assembled from whole genome sequences (WGS) of 809 Alzheimer Disease Neuroimaging Initiative (ADNI) cohort participants did not find any significant associations of AD risk or AD-related endophenotypes with mtDNA single nucleotide variants (SNVs) or MT haplogroups,[15] probably due to a small sample size.

Because the mitochondrial genome lacks introns and intergenic regions, except for the 1124 bp D-loop/control region, which is noncoding and contains the origin of replication and origin of transcription, we hypothesized that mtDNA genotypes can be deduced from whole-exome sequencing (WES) data with accuracy comparable to genotypes called from WGS data. Here, we report the development of a pipeline for calling mtDNA-variant genotypes and MT haplogroups from WES data obtained from nearly 11,000 subjects in the Alzheimer's Disease Sequencing Project (ADSP) Discovery Phase cohort and testing association of these variants and haplogroups, as well as with variants in nuclear genes that encode proteins involved in mitochondrial function, with AD risk.

2 | METHODS

2.1 | Participants

The ADSP performed WES of DNA specimens obtained from 5778 AD cases and 5136 controls, including 5519 AD cases and 4917 cognitively normal elderly controls of European ancestry (EA) and 218 AD cases and 177 controls of Caribbean Hispanic (CH) heritage. Notably, the proportion of AD cases with a known positive family history of dementia is much higher in the CH sample (78.9%) than the EA sample (9.2%). Detailed descriptions of the ADSP WES discovery phase study design and sequencing protocol have been published elsewhere.[16] After performing a series of filtering steps to identify duplicate samples and subjects with low genotype call rates, 10,436 EA and 395 CH individuals remained for further study. Subject characteristics are shown in Table 1 and described in detail elsewhere.[9]

2.2 | Whole exome sequencing, mitochondrial variant calling, and quality control

Details of library preparation, sequencing protocols, and autosomal nuclear variant calling pipelines were described previously.[9] In brief, 100 bp paired-end reads derived from cram files were

TABLE 1 Participant characteristics

		AD cases (N = 5737)					Cognitively normal controls (N = 5094)			
Ethnic group	Total N	N	N (%) enriched*	Mean Age	Female Sex (%)	*APOE* ε4 carrier (%)	N	Age (mean)	Female Sex (%)	*APOE* ε4 carrier (%)
European ancestry	10,436	5519	507 (9.2)	76.0	56.5	41.0	4917	86.5	59.1	12.8
Caribbean Hispanic	395	218	172 (78.9)	74.8	63.8	36.7	177	73.9	60.5	35.6

Abbreviation: *APOE*, apolipoprotein E.

RESEARCH IN CONTEXT

1. **Systematic review**: The authors are members of the Alzheimer's Disease Sequencing Project and therefore are familiar with emerging pertinent literature. PubMed searches were conducted to identify other relevant publications. References that support the significance of the identified risk loci are cited.
2. **Interpretation**: Although both common and rare variants in nuclear genome in > 30 late-onset Alzheimer's disease (LOAD) risk genes have been identified from genome-wide association and whole exome sequencing (WES) studies, this report first demonstrated that accurate mtDNA variants can be derived from a WES platform. Study-wide significant associations of AD with an MT gene (*MT-ND4L*) and an MT-related nuclear gene (*TAMM41*) were identified, providing further evidence for the role of mitochondria in AD.
3. **Future directions**: A better understanding of the molecular mechanisms underlying these associations will require functional experiment studies of the connections of genetic variants to mitochondrial dysfunction and further to AD-related neuropathogenesis. Further studies are also needed to determine whether *MT-ND4L* and *TAMM41* are suitable targets for development of novel therapies.

mapped to the human revised Cambridge Reference Sequence (rCRS) for human mitochondrial DNA (GenBank NCBI accession number: NC_012920/hg19)[17] using the Burrows-Wheeler Aligner (BWA).[18] The haploid mode implemented in the GATK 3.7 HaplotypeCaller package[19–21] was used to call mtDNA biallelic SNVs. We adapted the quality control (QC) protocols developed by the ADSP QC Working Group[22] to mtDNA SNVs to generate a high-quality variant call set. Because the Mitochondrial Chromosome (chrM) was not a WES capture target, the off-target read coverage in WES data of the MT genome is much less than that for the autosomal genome. This off-target mtDNA was still adequate due to the relatively high level of mtDNA relative to autosomal DNA. Therefore, we developed a pipeline for calling mtDNA variants and defined a QC metric based on comparative analysis with mtDNA variants called from WGS data for ADNI participants[15] and WGS data from the 1000 Genomes (1000G) reference panel.[23] Specifically, 4220 SNVs and small indels remained after excluding low-quality, multi-allelic, and monomorphic SNVs using filters of GQ < 20 and DP < 3 and a missing rate > 20%. After removing 182 subjects with missing values for all 4220 mtDNA variants, 10,610 subjects remained for haplogroup calling, downstream comparisons, and association analyses. Characteristics of these subjects are presented in Table 1. mtDNA variants were annotated using Gencode v24 (chrM.gencode.v24.annotation.gff3) and the Mitomaster sequence analysis tool within the MITOMAP human mitochondrial genome database.[24]

2.3 | Mitochondrial variant validation and comparison

To validate mtDNA variants called from the ADSP WES data, we applied the same calling pipeline to the ADNI WGS data (n = 809) and the 1000G WGS data (n = 2534). After calling and QC, we compared our mtDNA variants called from the WES data to that called from the WGS data from ADNI and 1000G. In addition, we compared our MT variants to known variants deposited in the MITOMAP database[24] and with 226 mtDNA variants genotyped in 4883 subjects in the Alzheimer's Disease Genetic Consortium (ADGC) using the Illumina Human Exome microarray.[25] Among 226 mtDNA variants in the exome chip, 174 were also called in the ADSP WES data. We verified the concordance of reference and alternative alleles for each of these mtDNA variants in 4883 subjects who were common to the ADGC and ADSP datasets.

2.4 | Mitochondrial haplogroup classification

HaploGrep2 software[26] was used to call phylogenetic clusters (haplogroups) from the filtered 4220 MT variants in the 10,610 subjects. The mtDNA haplogroups were classified with PhyloTree, Build 17, which comprises nearly 5500 haplogroups.[27]

2.5 | Association analysis methods

2.5.1 | Single mtDNA variant association analyses

Association of AD with each mtDNA variant having a minor allele count (MAC) ≥10 and call rate ≥ 0.8 was tested in each ethnic population (number of variants = 802 in EA and 135 in CH) using the *Score* test in seqMeta[28] with two additive logistic regression models as

previously described.[9] Model 1 included covariates for sequencing center and principal components (PCs) of ancestry (the first 10 PCs for EA and the 3 PCs for CH with $P < .1$ with association with AD) to identify variants whose effects on AD risk are confounded by age and sex in light of the unique ascertainment scheme for the WES sample.[9] Model 2 included these covariates and terms for age and sex. Results from analyses of 84 variants that were successfully called and passed criteria for single-variant analysis in the EA and CH data sets were combined using an inverse variance–weighted meta-analysis approach implemented in seqMeta. Bonferroni-corrected thresholds were applied to define study-wide significance (SWS) in each group (EA: $P < 6.20 \times 10^{-5}$, CH: $P < 3.70 \times 10^{-4}$, and meta $P < 5.90 \times 10^{-4}$).

2.5.2 | Gene-based association analysis

SNVs were annotated using a scheme developed by the ADSP Annotation Working Group and the HmtDB resource,[29] which hosts a database of human mitochondrial genome sequences from individuals with healthy and disease phenotypes[29] to discriminate variants predicted to have high or pathogenic functional impact on the protein product (i.e., HmtDB_Pathogenicity = "pathogenic"). Association was tested for genes with ≥ 2 variants and a cumulative MAC (cMAC) ≥ 10 after excluding variants with a minor allele frequency (MAF) ≥ 0.05 using the same models as in the individual variant analyses and the SKAT-O program in seqMeta.[28] Separate analyses were performed for the EA (16 genes) and CH (12 genes) groups. The ethnic-specific gene-based results were combined by meta-analysis of Z-scores weighted by the number of subjects using seqMeta, assuming the same direction of effect in both populations. Significance thresholds for each analysis were determined based on the number of genes tested in each group (EA: $P < 3.13 \times 10^{-3}$, CH: $P < 4.17 \times 10^{-3}$, and total: $P < 3.57 \times 10^{-3}$).

2.5.3 | mtDNA haplogroups association analysis

Association of AD with mtDNA haplogroups was tested separately in each ethnic group using a logistic regression model with covariates for age and sex.

2.5.4 | Gene-based association analysis of nuclear-encoded genes related to mitochondrial function

In light of evidence suggesting that nuclear genes involved in mitochondrial function are also associated with AD,[30] we tested the association of AD with 1158 nuclear-encoded genes with evidence of mitochondrial protein localization and protein distribution across 14 tissues identified from a public database MitoCarta2.0.[31] Because prior investigations of individual variants in these genes in the discovery dataset studied here[9] and in much larger samples[3,6] did not detect significant associations, we hypothesized that functional rare variants may contribute to AD risk and there is an increased chance to detect association with them using a burden test. Variants with predicted functional impact were selected and classified using the Ensembl Variant Effect Predictor (VEP)[32] and SnpEff[33] software. Variants annotated as splice acceptor, splice donor, stop gained, frameshift, stop lost, start lost, or transcript amplification were classified as high impact. These variants plus variants annotated as in-frame insertion, in-frame deletion, missense variant, or protein altering were classified as high or moderate impact. Association was tested for each gene using the approach described in Section 2.5.2. Significance thresholds for each analysis were determined based on the number of genes tested in each group (high impact variants—EA: $P < 2.30 \times 10^{-4}$, CH: $P < 5.56 \times 10^{-3}$, and total sample: $P < 3.30 \times 10^{-4}$; high or moderate impact variants—EA: $P < 5.03 \times 10^{-5}$, CH: $P < 7.49 \times 10^{-5}$, and total sample: $P < 5.48 \times 10^{-5}$).

2.6 | Bioinformatics analysis methods

Differential gene expression (DGE) and network analyses were performed for 1171 protein-coding genes (13 MT and 1158 autosomal) related to mitochondrial function using RNAseq data derived from the dorsolateral prefrontal cortex (DLPFC) of 634 participants (210 controls, 167 mild cognitive impairment [MCI] cases, and 257 AD cases) of the Religious Orders Study and Rush Memory and Aging Project (ROSMAP). RNAseq data were obtained from the AMP-AD Knowledge Portal (Synapse: syn3388564). Reads were mapped to the human reference sequencing (hg38) using STAR v2.4.2a[34] and expression of protein-coding genes was quantified using RESM v1.2.29[35] with Gencode 28 (Ensembl 92) gene annotation. After filtering out genes with low expression level determined as the average of log (counts per million reads) > 1, differential expression was evaluated for 13,650 protein-coding genes using Deseq2.[36] The association of differential gene expression with clinical outcome was evaluated in pairwise comparisons of AD, MCI, and control subjects using regression models including covariates for age, sex, and *post mortem* interval (PMI). Gene coexpression networks were constructed using weighted gene coexpression network analysis (WGCNA)[37] across all 635 samples, and the 13 MT genes and 1158 nuclear genes involved in MT served as input for these analyses. Association of significant modules identified by WGCNA with AD status and several AD-related endophenotypes including Braak stage and neuritic plaque density was evaluated by eigenvalues derived from each module.

2.7 | Polygenic risk scores

Polygenic risk scores (PRS) for AD were calculated using summary results for single nucleotide polymorphisms (SNPs) with a *P*-value less than 1.0×10^{-5} obtained from a recent large AD GWAS.[6] Linkage disequilibrium (LD) pruning was performed to exclude SNPs that were correlated ($r^2 > 0.5$) with another variant with smaller *P*-value within a 250 kb window. SNPs were weighted by their effect sizes (beta value) in the GWAS. A total of 226 LD-pruned SNPs was included in the calculation of the PRS for 221 ROSMAP subjects having both GWAS

and RNA-seq expression data. The PRS was tested for association with the eigenvalue derived from each significant module.

3 | RESULTS

3.1 | MT DNA variant calling, validation, and comparison

We identified 4220 high-quality mtDNA SNVs in the ADSP WES dataset (GQ > 20, DP > 3, call rate ≥ 0.8). Using the same calling and QC pipeline, 1851 mtDNA variants were called in the ADNI WGS dataset[15] and 3892 mtDNA variants were called in the 1000G WGS dataset.[23] The mtDNA variants identified in the WES dataset included 84% (1548/1851) of the total found in the ADNI dataset. Of the 1548 variants common to both datasets, 1332 (86%) also matched at allele level (i.e., reference and alternate alleles). Similarly, the set of mtDNA variants identified in the WES dataset included about 68% (2628/3892) of the total variants present in the 1000G dataset and 83% of the variants common to both datasets (2169/2628) also matched allele level. In addition, the WES dataset contained 3620 of the 3855 (94%) of biallelic mtDNA variants in the MitoMap database.[24] To further validate the accuracy of our mtDNA variant calling pipeline, we compared genotypes for 174 mtDNA variants determined for 4883 subjects to both WES and Exome Chip data. The concordance for the reference allele was 99.65% to 99.98% for 20 variants and 100% for the remaining 154 variants (Table S1 in supporting information). One alternate allele that was observed in one subject in the exome chip dataset was not called in the WES dataset. The concordance of minor alleles was 50% to 86% for 10 variants, 90% to 99% for 12 variants, 99.0% to 99.9% for 20 variants, and 100% for 112 variants. An additional 19 variants were monomorphic in both datasets.

A total of 16 major MT haplogroups were called by HaploGrep2 using the same human MT reference genome sequence (NC_012920; Table S2 in supporting information). Among EAs, Haplogroup I was nominally associated with AD (odds ratio [OR] = 1.37, $P = .02$), but this result was not significant after adjusting for the number of haplogroup tests. The MT haplogroup frequencies differ between the CH and EA samples, reflecting the ancestral admixture of African, European, and Native American populations of Caribbean populations. L, which is the most common haplogroup in the CH sample (frequency = 0.5), is the African ancestral MT haplogroup. While there is a modest association of AD with the two ancestral Native American haplogroups (B and C, $P < .04$) in the CH sample, these results are not significant after multiple-test correction (Table S3 in supporting information).

3.2 | Association of AD with MT variants and genes

In the combined EA and CH sample, AD was significantly associated with missense mutation rs28357675 (Asn119Ser) in *MT-ND6* ($P = 5.3 \times 10^{-4}$) and synonymous variant rs193302991 in *MT-CYB* ($P = 5.14 \times 10^{-4}$) after adjusting for age and sex (Table 2). These results

TABLE 2 MT single variant results

					European ancestry				Caribbean Hispanic				Total		
Top SNV	rsID	Function	Gene	Model	MAF (%)	MAC	β (se)	P value	MAF (%)	MAC	β (se)	P value	MAC	β (se)	P value
26:10733:C:T	rs28709356	*Synonymous*	*MT-ND4L*	M1	0.30	26	0.96 (0.39)	.01	0	NA	NA	NA	NA	NA	NA
				M2	0.30	26	2.02 (0.51)	7.30×10^{-5}	0	NA	NA	NA	NA	NA	NA
26:14318:T:C	rs28357675	*Missense* Asn119Ser	*MT-ND6*	M1	0.24	12	1.07 (0.59)	.07	4.42	17	1.59 (0.52)	2.50×10^{-3}	29	1.36 (0.39)	5.25×10^{-4}
				M2	0.24	12	0.94 (0.77)	.23	4.42	17	1.59 (0.53)	2.50×10^{-3}	29	1.38 (0.44)	1.48×10^{-3}
26:15301:G:A	rs193302991	Synonymous	*MT-CYB*	M1	1.20	109	0.46 (0.19)	.02	44.9	162	0.59 (0.23)	.01	271	0.52 (0.15)	5.98×10^{-4}
				M2	1.20	109	0.55 (0.25)	.02	44.9	162	0.62 (0.23)	7.86×10^{-3}	271	0.59 (0.17)	5.14×10^{-4}

Model 1 (M1) = AD ~ Center + PCs + SNV; Model 2 (M2): AD ~ Center + PCs + age + sex.
Study-wide significance threshold was defined by 0.05/the number of variants tested. EA: $P < 6.2 \times 10^{-5}$, CH: $P < 3.7 \times 10^{-4}$, total: $P < 5.9 \times 10^{-4}$.
Abbreviations: AD, Alzheimer's disease; EA, European ancestry; CH, Caribbean Hispanic; MT, mitochondrial; PCs, principal components; SNV, single nucleotide variants.
[Correction added on August 10, 2021 after first online publication: The first value under the "Function" column was revised from "Missense Asp88Glu".].

TABLE 3 Mitochondrial gene-based results

		European ancestry			Caribbean Hispanic			Total		
Gene	Model	# SNPs	cMAC	*P*-value	# SNPs	cMAC	*P*-value	# SNPs	cMAC	*P*-value
MT-ND4L	M1	14	89	.04	3	5	NC	14	94	.06
	M2	14	89	9.36×10^{-5}	3	5	NC	14	94	6.71×10^{-5}
MT-ND2	M1	49	460	.08	14	101	8.0×10^{-3}	53	561	.02
	M2	49	460	.32	14	101	8.3×10^{-3}	53	561	.09
MT-ND5	M1	159	2741	.02	25	387	.47	165	3128	3.30×10^{-3}
	M2	159	2741	.37	25	387	.41	165	3128	.06

Model 1 (M1) = AD ~ Center + PCs + Gene; Model 2 (M2): AD ~ Center + PCs + Gene + age + sex.
Study-wide significance threshold was defined by 0.05/the number of genes tested: EA: $P < 3.13 \times 10^{-3}$, CH: $P < 4.17 \times 10^{-3}$, total: $P < 3.57 \times 10^{-3}$.
Abbreviations: AD, Alzheimer's disease; CH, Caribbean Hispanic; cMAC, cumulative minor allele count; EA, European ancestry; MT, mitochondrial; NC, not calculated because of an insufficient number of minor alleles; PCs, principal components; SNPs, single nucleotide polymorphisms.

TABLE 4 Gene-based results for nuclear-encoded genes related to mitochondrial function

			European ancestry		Caribbean Hispanic		Total	
Variant impact	Gene	Model	# SNPs	*P*-value	# SNPs	*P*-value	# SNPs	*P*-value
High	*GPD2*	M1	5	2.70×10^{-4}	NA	NA	5	3.70×10^{-4}
		M2	5	.005	NA	NA	5	.0062
High/moderate	*TAMM41*	M1	30	.0025	6	.95	34	.0075
		M2	30	2.70×10^{-5}	6	.91	34	4.60×10^{-4}
	GPT2	M1	35	.27	4	4.00×10^{-3}	38	.05
		M2	35	.51	4	3.90×10^{-3}	38	.06

Model 1 (M1) = AD ~ Center + PCs + Gene; Model 2 (M2): AD ~ Center + PCs + Gene + age + sex.
Study-wide significance threshold was defined by 0.05/the number of genes tested.
High impact variants: EA: $P < 2.3 \times 10^{-4}$, CH: $P < 5.56 \times 10^{-3}$, total: $P < 3.30 \times 10^{-4}$.
High/moderate impact variants: EA: $P < 5.03 \times 10^{-5}$, CH: $P < 7.49 \times 10^{-5}$, total: $P < 5.48 \times 10^{-5}$.
Abbreviation: SNPs, single nucleotide polymorphisms.

were more significant in the relatively small CH sample, an observation that may be explained by the higher min or allele count for each of these variants in that group. A near study-wide significant result (OR = 7.52; $P = 7.3 \times 10^{-5}$) was observed in EAs with a rare *MT-ND4L* variant (rs28709356 C>T, MAF = 0.002). [Correction added on August 10, 2021 after first online publication: The preceding sentence was revised from, "... was observed in EAs with the MT-ND4L Asp88Glu missense mutation (rs28709356 MAF=0.002). This mutation is predicted to be deleterious (SIFT score=0.004)."] *ND4L* is highly expressed in multiple brain regions (Figure S1 in supporting information). Gene-based tests focused on pathogenic/high-impact variants revealed that *MT-ND4L* was SWS in EA ($P = 9.36 \times 10^{-5}$) and in the total sample (meta $P = 6.71 \times 10^{-5}$) under Model 2 (Table 3). The association with *MT-ND5* was also SWS in the total sample (meta $P = 3.3 \times 10^{-3}$). None of the 16 MT haplogroups identified in the sample were associated with AD after multiple test correction (Table S2).

3.3 | Association results of nuclear-encoded mitochondrial genes

Of the 1158 nuclear genes encoding proteins related to mitochondrial function, 217 genes in EAs and nine genes in the CH group contained multiple high-impact variants. None of the tests with these genes were SWS; however, in the model without adjustment for age and sex (Model 1), *GPD2* approached the SWS threshold in the EA sample ($P = 2.7 \times 10^{-4}$) and combined EA+CH groups ($P = 3.7 \times 10^{-4}$, Table 4A). In analyses that included high- and moderate-impact variants, SWS association was observed with *TAMM41* ($P = 2.7 \times 10^{-5}$) in the EA group for the model adjusting for age and sex (Table 4B). None of the gene-based tests were SWS in the CH group, probably because of the small sample size.

3.4 | Functional analysis results

Analysis of the ROSMAP RNAseq data derived from the DLPFC region showed that expression of *TAMM41* is lower in AD cases than controls ($P = .00046$) or MCI cases ($P = .03$), but not different between MCI cases and controls ($P = .25$). The expression of *GPT2* is higher in AD cases than controls ($P = .00047$), but not different from MCI cases ($P = .10$) and between MCI cases and controls ($P = .14$, Table 5). None of the MT genes are differentially expressed between AD cases and controls; however, trends of increased expression of three of these

TABLE 5 Differential expression of TAMM41 and GPT2 in dorsolateral prefrontal cortex of 634 ROSMAP subjects

	TAMM41			*GPT2*		
Comparison Groups*	Base mean	Log2 fold change	*P*-value	Base mean	Log2 fold change	*P*-value
AD vs. Normal	134	−0.11	.00046	2419	0.18	.00047
AD vs. MCI	131	−0.07	.03	2477	0.09	.10
MCI vs. Normal	134	−0.04	.25	2419	0.08	.14

Abbreviations: AD, Alzheimer disease; MCI, mild cognitive impairment.
*Sample sizes: AD = 257; MCI = 167, normal = 210.

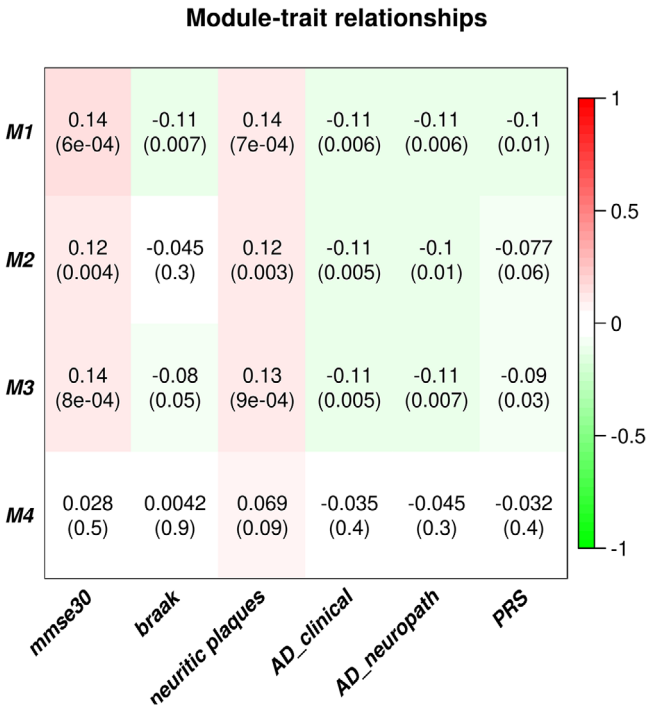

FIGURE 1 Heatmap of module-trait relationships depicting correlations between module eigengenes and clinical/pathological Alzheimer's disease (AD) status and AD-related endophenotypes traits. Numbers in the table correspond to the coefficient and *P*-value (in parentheses) for the pairwise correlations. The degree of correlation is illustrated with the color legend. Note that increasing Mini-Mental State Examination (MMSE) and plaque scores, and decreasing values for Braak stage and AD status, are in the direction of normal

genes (*MT-ND5*, *MT-ATP8*, and *MT-CO1*) in AD cases approach nominal significance ($P < .06$, Table S4 in supporting information).

Co-expression network analysis of 13 MT-encoded and 1158 nuclear-encoded mitochondrial genes revealed four co-expression modules (Figure 1). Three of these modules were also significantly associated with the CERAD neuritic plaque score, particularly Module 1 ($P = 7.0 \times 10^{-4}$) and Module 3 ($P = 9.0 \times 10^{-4}$). Module 1 is also associated with Braak stage ($P = .007$), clinical and neuropathological AD status ($P = .006$), and Mini-Mental State Examination score ($P = 6.0 \times 10^{-4}$). Gene Ontology (GO) analysis of these four modules revealed significant enrichment in Module 1 of 168 MT-related genes as expected of genes involved in mitochondrial functions, as well as for genes with roles in several neurodegenerative disorders including AD ($P < 1.95 \times 10^{-10}$, Table 6). The PRS for AD was significantly associated with Module 1 ($P = .01$) and module 3 ($P = .03$, Figure 1).

4 | DISCUSSION

Numerous studies indicate that mitochondrial dysfunction may portend AD-related brain pathology,[38] and mitochondrial genes are altered in blood in early-stage AD.[39] Emerging evidence suggests a role for mitochondria in synaptic transmission and neurodegeneration, and the ability of dysfunctional mitochondria to trigger apoptosis.[40] A recent study demonstrated that healthy mitochondria can halt amyloid beta (Aβ) proteotoxic diseases, such as AD, as increasing mitochondrial proteostasis reduces amyloid aggregation in cells, worms, and in transgenic mouse models of AD.[41]

To determine whether mtDNA mutations may influence the pathogenesis of AD, we developed a pipeline for identifying mtDNA variants in WES data and assessing the quality of MT genotype calls. To validate our pipeline and mtDNA variants called, we compared genotypes in the ADSP WES dataset to those derived from ADNI and 1000G reference panel WGS datasets and genotypes for the ADGC dataset obtained using an exome microarray chip. The mtDNA genotypes obtained from these various sources were very similar, suggesting that mitochondrial variants and haplogroups can be reliably derived from WES data. Using this pipeline, we derived a set of high-confidence mtDNA genotypes and haplogroups from a WES dataset comprised of 5737 AD cases and 5094 controls from the ADSP.

Analysis of these data revealed in the relatively large EA portion of the sample association of AD with a rare synonymous mutation (rs28709356, Asp88Asp) in *MT-ND4L* as well as with an aggregate of 14 *MT-ND4L* SNVs in a gene-based test. [Correction added on August 10, 2021 after first online publication: The preceding sentence was revised from, "... with a rare missense deleterious mutation (rs28709356, Asp88Glu) in MT-ND4L...".] In the total sample, we found association with rare variants in *MT-ND6* and *MT-CYB*, results accounted for primarily by the much smaller CH sample. A SWS association was also detected by gene-based testing with *MT-ND5* in the total sample. In contrast, few previous reports of association of mitochondrial haplogroups and SNPs with AD risk and cognitive function in datasets much smaller than this study have been replicated.[42–44] There is some evidence suggesting that interactions between mitochondrial genetic variation and apolipoprotein E (*APOE*) genotype influences AD risk.[45,46] We also showed that a PRS for AD derived from nuclear SNP results obtained by a large AD GWAS was associated with an

TABLE 6 Gene Ontology enrichment analysis results using 168 MT-related genes in Module 1

Term	Count	%	*P*-value	Genes	Adjusted P-value
GO:0070125~mitochondrial translational elongation	18	10.98	1.20E-18	MRPL53, MRPL52, MRPS26, MRPS16, MRPS34, MRPL4, MRPL41, AURKAIP1, MRPS12, MRPS24, MRPL20, GADD45GIP1, MRPL12, MRPL28, MRPL54, MRPL55, MRPL38, MRPL34	6.70E-16
GO:0070126~mitochondrial translational termination	18	10.98	1.49E-18	MRPL53, MRPL52, MRPS26, MRPS16, MRPS34, MRPL4, MRPL41, AURKAIP1, MRPS12, MRPS24, MRPL20, GADD45GIP1, MRPL12, MRPL28, MRPL54, MRPL55, MRPL38, MRPL34	8.28E-16
GO:0032981~mitochondrial respiratory chain complex I assembly	15	9.15	3.05E-16	NDUFV3, NDUFS7, NDUFS6, NDUFB11, NDUFA3, NDUFB10, NDUFAF8, NDUFB7, NDUFV1, NDUFS8, NDUFA13, ECSIT, NDUFB1, NDUFA11, NDUFAF3	1.86E-13
GO:0003735~structural constituent of ribosome	22	13.41	4.32E-16	MRPL52, MRPS16, MRPL4, MRPS34, MRPL41, SLC25A6, MRPS12, MRPS24, MRPL20, SLC25A11, MRPL12, MRPL28, SLC25A10, SLC25A22, MRPL55, SLC25A1, MRPL57, SLC25A45, SLC25A39, SLC25A42, MRPL34, SLC25A41	1.09E-13
GO:0006412~translation	21	12.80	1.24E-13	MRPL52, MRPS16, MRPL4, MRPL41, PDF, SLC25A6, MRPS12, MRPS24, MRPL20, SLC25A11, MRPL28, SLC25A10, SLC25A22, MRPL55, SLC25A1, MRPL57, SLC25A45, SLC25A39, SLC25A42, MRPL34, SLC25A41	6.90E-11
hsa05012: Parkinson's disease	17	10.37	1.64E-13	NDUFB11, NDUFB10, NDUFA3, NDUFB7, SLC25A6, COX8A, CYC1, NDUFA13, COX5B, NDUFB1, NDUFA11, NDUFV3, NDUFS7, NDUFS6, UQCR11, NDUFV1, NDUFS8	1.38E-11
GO:0006120~mitochondrial electron transport, NADH to ubiquinone	12	7.32	4.67E-13	NDUFV3, NDUFS7, NDUFS6, NDUFB11, NDUFB10, NDUFA3, NDUFB7, NDUFV1, NDUFS8, NDUFA13, NDUFB1, NDUFA11	2.60E-10
hsa00190: Oxidative phosphorylation	16	9.76	1.03E-12	NDUFB11, NDUFB10, NDUFA3, NDUFB7, COX8A, CYC1, NDUFA13, COX5B, NDUFB1, NDUFA11, NDUFS7, NDUFV3, NDUFS6, UQCR11, NDUFV1, NDUFS8	8.67E-11
hsa05010: Alzheimer's disease	17	10.37	2.32E-12	NDUFB11, NDUFB10, NDUFA3, NDUFB7, COX8A, CYC1, NDUFA13, BAD, COX5B, NDUFB1, NDUFA11, NDUFS7, NDUFV3, NDUFS6, UQCR11, NDUFV1, NDUFS8	1.95E-10
hsa04932: Non-alcoholic fatty liver disease (NAFLD)	16	9.76	6.75E-12	NDUFB11, NDUFB10, NDUFA3, NDUFB7, COX8A, CYC1, NDUFA13, COX5B, NDUFB1, NDUFA11, NDUFS7, NDUFV3, NDUFS6, UQCR11, NDUFV1, NDUFS8	5.67E-10
hsa05016: Huntington's disease	17	10.37	1.83E-11	NDUFB11, NDUFB10, NDUFA3, NDUFB7, SLC25A6, COX8A, CYC1, NDUFA13, COX5B, NDUFB1, NDUFA11, NDUFV3, NDUFS7, NDUFS6, UQCR11, NDUFV1, NDUFS8	1.54E-09
GO:0008137~NADH dehydrogenase (ubiquinone) activity	10	6.10	3.00E-10	NDUFV3, NDUFS7, NDUFS6, NDUFB10, NDUFA3, NDUFB7, NDUFV1, NDUFS8, NDUFA13, NDUFB1	7.36E-08
hsa01100: Metabolic pathways	33	20.12	8.63E-09	PTGES2, BCAT2, NDUFB7, CYC1, AGMAT, COX5B, NDUFB1, NDUFS7, NDUFS6, UQCR11, NT5M, NDUFS8, DHODH, FASN, NT5C, NDUFB11, NDUFA3, NDUFB10, ACADS, COX8A, MCAT, NDUFA13, NDUFA11, NDUFV3, TST, NME4, PYCR2, NME3, NDUFV1, FPGS, GUK1, DCXR, MPST	7.25E-07
IPR018108: Mitochondrial substrate/solute carrier	9	5.49	1.34E-08	SLC25A11, SLC25A10, SLC25A6, SLC25A22, SLC25A1, SLC25A39, SLC25A45, SLC25A42, SLC25A41	4.98E-06
IPR023395: Mitochondrial carrier domain	9	5.49	1.34E-08	SLC25A11, SLC25A10, SLC25A6, SLC25A22, SLC25A1, SLC25A39, SLC25A45, SLC25A42, SLC25A41	4.98E-06
IPR002067: Mitochondrial carrier protein	7	4.27	8.19E-08	SLC25A6, SLC25A22, SLC25A1, SLC25A39, SLC25A45, SLC25A42, SLC25A41	3.05E-05
GO:0003954~NADH dehydrogenase activity	5	3.05	3.86E-07	NDUFS7, NDUFV1, NDUFS8, NDUFA13, NDUFB1	9.47E-05
GO:0032543~mitochondrial translation	6	3.66	1.66E-05	MRPS16, MRPS34, PTRH1, MRPS12, MRPS24, MRPL57	0.0092
GO:0003824~catalytic activity	10	6.10	4.22E-05	ECI1, D2HGDH, BCAT2, DHODH, FASN, GCAT, ISOC2, PMPCA, NTHL1, ACSF3	0.010

AD-related gene coexpression module enriched for MT genes, thus providing insights about the joint contributions of variation in mitochondrial genes and nuclear-encoded genes related to mitochondrial function to AD. This observation is consistent with a recent ADNI study which found association of AD with interactions of particular mitochondrial DNA haplogroups and a PRS derived from nuclear-encoded mitochondrial genes.[47]

MT-ND4L, *MT-ND6*, *MTND2*, and *MT-ND5* encode subunits of complex I (NADH dehydrogenase), and are active in metabolic pathways and oxidative phosphorylation. There is some evidence suggesting that impairments in complex I enzyme activities and subunit assembly are involved in AD.[43,48,49] Aβ peptide alters the enzyme activity of complex I, and mitochondrial functions can be negatively affected by Aβ.[50] A recent multivariate meta-analysis concluded that complex I and IV enzymes are deficient in AD.[51] *MT-ND4L* encodes the mitochondrial NADH dehydrogenase subunit 4L involved in ubiquinone activity and oxidoreductase activity. Rare *MT-ND4L* SNVs have been associated with bipolar disorder, major depression, and Leber's optic atrophy.[52–54] MT-ND2 was previously been associated with AD[55] and a *MT-ND6* variant was associated with a significant decline in cognitive function.[43] *MT-CYB*, which encodes a complex III subunit, has not been previously linked to AD.

We also tested association of AD with functional variants in 1158 nuclear genes that encode proteins involved in mitochondrial function. Although no significant findings were identified with any individual variants in these genes, SWS association was observed with a collective group of SNVs in *TAMM41* in a gene-based test. In addition, we showed that expression of *TAMM41* was higher in brains from AD cases than MCI cases, suggesting a stage-dependent indicator for conversion to AD. The gene product of *TAMM41*, mitochondrial translocator assembly and maintenance protein 41 homolog, is a mitochondrial membrane maintenance protein and is required for the biosynthesis of phospholipid, CDP-diacylglycerol, cardiolipin, and phosphatidylinositol (PI).[56] It has been shown that selectively inhibiting Aβ-induced PI-4,5-bisphosphate (PIP2) hydrolysis in the CA3 region of the hippocampus strongly prevents oligomeric Aβ-induced suppression of prion protein at the SC-CA1 synapse and rescues synaptic and spatial learning and memory deficits in APP/PS1 mice.[57]

Several strengths and limitations of our study warrant discussion. To our knowledge, this is the first large study of rare MT genetic variants in a sample of carefully clinically and genetically characterized AD cases and elderly cognitively healthy controls. One limitation of the study is that the sample included a comparatively small number of CH participants (N = 396) and thus there was little power to detect associations with rare variants in this group. In addition, our study did not evaluate association of AD with individual variants in nuclear-encoded mitochondrial genes because these tests have already been performed in this dataset[9] and for common variants in much larger GWAS datasets that include subjects in this study[3,6] without any significant results. Instead, we evaluated the effects of aggregated rare variants in and differential expression between AD cases and controls in these genes. This strategy yielded significant associations with three genes (*GPD2*, *TAMM41*, and *GPT2*), and two of them (*TAMM41* and *GPT2*) showed significant differential expression. We also recognize that our findings should be replicated in independent AD WES or WGS samples that are sufficiently large to detect associations with rare variants, noting that approximately one-half of our significant results were observed only in or primarily due to the CH dataset. Finally, due to the low abundance of reads mapping to the MT genome in WES data, it is challenging to estimate accurately MT heteroplasmy and MT copy number (i.e., the number of copies of the MT genome within a cell). Because both have been linked to aging[30] and several neurodegenerative diseases,[58,59] future research should focus on quantifying MT heteroplasmy and MT copy number variation, and testing their association with AD risk using high coverage whole genome sequence data (i.e., > 30X) from multi-ethnic cohorts.

In summary, we called mtDNA variants in a large WES dataset from the ADSP with a level of confidence comparable to that for variants called from WGS data or genotyped directly on SNP arrays. We identified significant association of AD risk with individual and aggregated rare mtDNA variants in *MT-ND4L* and a nuclear-encoded MT gene, *TAMM41*, suggesting variants in MT or nuclear genes leading to mitochondrial dysfunction may be related to AD risk. Findings from our work and other relevant studies[60,61] indicate that a better understanding of the molecular mechanisms underlying these associations will require functional experiments and *in silico* studies of the connections of MT genetic variants to gene expression, processing of AD-related proteins, and mtDNA epigenetic modulation in human brain.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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