Cell-Free mtDNA Quantification in Alzheimer's Patients from the Mexican American Population

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Abbreviation	Full Name
CF-mtDNA	Cell-Free mitochondrial DNA
AD	Alzheimer's Disease
MCI	Mild Cognitive Impairment
NC	Normal Control
TARCC	Texas Alzheimer's Research and Care Consortium
MMSE	Mini-Mental State Examination
CDR-SOB	Clinical Dementia Rating sum of boxes
OXPHOS	Oxidative-Phosphorylation
ETC	Electron Transport Chain
ROS	Reactive Oxygen Species
RNS	Reactive Nitrogen Species
T2D	Type 2 Diabetes
HBV	Hepatitis B Virus
MDV	Mitochondrial Derived Vesicles
DAMP	Damaged-associated Molecular Patterns
TNF-α	Tumor Necrosis Factor alpha
IL-8	Interleukin-8
NFκB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
NINCDS-ADRDA	National Institute of Neurological and Communicative Disorders and Stroke - Alzheimer's Disease and Related Disorders Association
BMI	Body Mass Index
q-PCR	Quantitative Polymerase Chain Reaction
HPLC	High Performance Liquid Chromatography
NFQ	Non-Fluorescent Quencher
IPC	Internal Positive Control
C_T	Cycle Threshold
SD	Standard Deviation
ANOVA	Analysis of Variance
CI	Cognitive Impairment
NIH/NIMHD	National Institute of Health / National Institute on Minority Health and Health Disparities

Abstract

Background

AD is a continuous problem in the 65+ population but it is especially challenging in the Hispanic population where not only is it more prevalent but more severe than Caucasian populations. This study explores the efficacy of using peripheral blood plasma as an alternative tissue for testing as well as the usefulness for future research assisting in identifying the population structure most at risk for developing AD based upon CF-mtDNA quantity results.

Materials and Methods

Samples tested included a total cohort (Mexican American and Caucasian) of 177 individuals (AD=45, MCI=74, NC=58). The Mexican American subset contained 92 individuals (AD=21, MCI=53, and NC=18). Peripheral blood plasma was collected from the TARCC biobank and quantified. CF-mtDNA was then tested for significance using correlation analyses, logistic and linear regression models.

Results

CF-mtDNA was significantly negatively correlated with education, age, sex, and hypertensive samples in the total and Mexican American populations. The greatest difference was expected to be in CF-mtDNA quantity from NC to AD samples. Instead, the most significant difference was between MCI and NC samples. As CF-mtDNA quantity increased, the MMSE and CDRSOB scores were less impaired.

Conclusion

In conclusion, CF-mtDNA is an easily accessible and easily tested molecular marker of diseases that are relevant to studies for cognitive decline. Although our findings were inconsistent with current literature, they bring to light the weight of confounding factors within limited sample studies. With the completion of the full sample set associated with this study, more power is needed to overcome these issues.

Chapter 1: Background

Mitochondrial DNA (mtDNA) reduction in cells has been correlated with mitochondrial dysfunction as an early indicator of Alzheimer's disease (AD) and some of its comorbidities, such as diabetes[1]. While brain mtDNA would be ideal for quantifying and assessing mitochondrial dysfunction in neurodegenerative diseases, sample size and the inability to sample living subjects becomes an issue. Cell-Free mtDNA(CF-mtDNA) is mtDNA that is circulating within the bloodstream[2 3], specifically found in the serum or plasma, originating from a variety of tissues, and; therefore, represents a vastly different biological phenomenon from mtDNA copy number which is the number of mtDNA genome copies found per cell[3 4]. CF-mtDNA reflects systemic communication, as opposed to indicating mitochondrial biogenesis, due to the mechanisms by which mtDNA are sent to the extracellular space (i.e., altered mitophagy and endolysosomal trafficking). This project investigates if CF-mtDNA is associated with cognitive phenotypes in the Texas Alzheimer's Research and Care Consortium (TARCC), an aging cohort with a spectrum of cognition ranging from normal, to Mild Cognitive Impairment (MCI), to AD.

Cell-free mitochondria in aging

The mitochondria are cellular organelles found in eukaryotes that are responsible for performing Oxidative-Phosphorylation (OXPHOS), a process that generates ATP as energy to be used in various cellular functions[5], by way of the Electron Transport Chain (ETC). This generation of ATP is also accountable for the production of reactive oxygen and nitrogen species (ROS, RNS) which have been linked to mitochondrial dysfunction over time and has been implicated in accelerated aging and age-related diseases. This degradation is reflected through mtDNA copy number reduction within the implicated cells. Recent studies have discussed using mtDNA extracts

from the buffy coat[6], the layer containing white blood cells and platelets, as a biomarker for diseases such as Type II Diabetes (T2D) and Hepatitis B Virus (HBV)[7], but the use of peripheral blood plasma extracts has yet to be used as the sole indicator for Alzheimer's disease within the Mexican American population, who have a different set of age-related comorbidities when compared to non-Hispanic Caucasians.

The first question to consider is, how is mtDNA circulating? Clearly, after they have entered a high stress state, mtDNA can escape the cell through a variety of pathways, in concordance with mitochondrial quality control standards, such as autophagy or mitophagy, to end up in extracellular release, shown in Figure 1. These two processes differ in that autophagy is a cellular self-eating mechanism by which intracellular elements are taken up and degraded by lysosomes[8 9] and mitophagy is the specific selection and removal of mitochondria through mitochondrial derived vesicles (MDV) when there has been a loss of mitochondrial membrane potential[9 10]. There have been many studies associating circulating CF-mtDNA acting as mitochondria-derived damage-associated molecular patterns (DAMPs)[9] by speeding up the aging process through a series of chronic increases in pro-inflammatory responses such as macrophage activation or in later pro-inflammatory signaling proteins such as TNFα, IL-8, and NFκB [11 12]. This phenomenon has been dubbed "Inflamm-aging"[13] and it has become a central theme of mtDNA research[6 9 11 14-16].

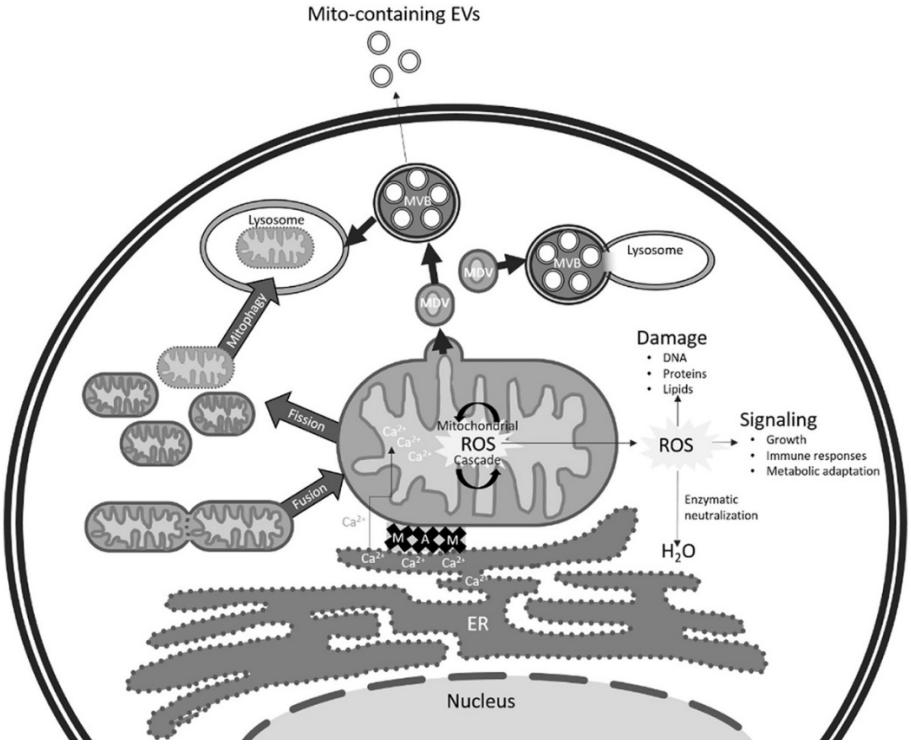

Fig. 1. Diagram of the various mitochondrial processes generally implicated in Alzheimer's disease. Mitochondria are components in many complex processes, from controlling cellular respiration (OXPHOS) to homeostasis (ion regulation, fission/fusion) and cellular signaling (ROS). While mitochondrial quality control measures are in place to circumvent both ROS and the resulting loss of function (collecting faulty components into MDVs, mitophagy, autophagy), damage to mtDNA can continue the cycle. This is the continuous cycle known as The Mitochondrial Cascade Hypothesis[17]. The cascade results in the damaged components interfering with pro-inflammatory responses by becoming mitochondria-derived DAMPs in the extracellular matrix. Image from Silzer et al., 2018.

Alzheimer's Disease

This study was funded by the Texas Alzheimer's Research and Care Consortium (TARCC) which is a collaborative research organization with the top 10 medical research institutions in Texas that has created the mission to work "to improve early diagnosis, treatment, and prevention of Alzheimer's disease and related brain disorders"[18]. In the United States Alzheimer's Disease is the 6th leading cause of death and there are currently more than 5.8 million people with this disease. By the year 2050, this number is expected to rise to over 14 million people affected[19].

Unfortunately, AD is only one subtype of dementia cases although it does account for more than two-thirds of all cases[19]. AD is a continuous problem in the 65+ population but it is especially challenging in the Hispanic population where not only is it more prevalent but more severe than Caucasian populations[20]. With these disparities now realized in the Mexican American population, early diagnosis is paramount for any possible interventions to slow the progression of the disease.

AD is described as having an overproduction of the beta-amyloid protein that results in the formation of amyloid plaques (AB plaques) in the space between nerve cells[21 22]. Because these plaques had already been implicated in trisomy 21 Down's Syndrome[23 24], which has a very similar neurocognitive presentation as AD, it was logical to look into this as being the cause for AD as well. In this line of research reasoning, tau tangles were discovered[22]. Tau tangles are neurofibrillary tangles of the tau protein within nerve cells and had been associated with Parkinson's Disease, but they have not been solely implicated as a cause of AD[21 25]. This change in tau pathology led research to study its contribution to mitochondrial dysfunction[26 27] and, subsequently, development of the mitochondrial cascade hypothesis (Figure 1). The presence of mitochondrial dysfunction would indicate the potential of AD or MCI long before the generation of plaques or tangles[25].

Comorbidities with AD

Considering AD is a chronic and lethal disease, it is important to use possible comorbidities and biomarkers as early indicators[28]. In the case of AD, key comorbidities, particularly in the Mexican American population, are T2D, obesity, and hypertension. If a patient is diagnosed with one or

more of these secondary diseases, they are at an increased risk of developing AD. With T2D specifically, the risk of developing AD increases by 50%[29 30]. Obesity is major correlating factor of adult diabetes, T2D[31-33]. The Mexican National Health and Nutrition Survey reported that 69% of male individuals are categorized as overweight and 76% of female individuals are overweight, regardless of age[34]. This is highly significant because Mexico has the second-highest obesity rate in the world (after the United States)[35]. Mexican American adults have more than a 50% chance of developing T2D within their lifetime. Along with the inherent increased diagnosis, Mexican Americans are also more likely to develop T2D at a younger age[35]. Mexican Americans on average have a 10-year younger age of onset than non-Hispanic white counterparts [7], and the prevalence of T2D in Mexican Americans is likely a key factor in this health disparity. This is further evidenced by the following two facts: (1) the genetic factors which drive risk for AD in Mexican Americans are not the same as in non-Hispanic whites, who have a highly APOε4 dependent risk [36-38]; and (2) Mexican Americans' blood-based proteomics profile, which is predictive of AD in this population, is unique when compared to non-Hispanic whites and features a more metabolic signature [7].

Limitations of detecting mtDNA quantity variance

The purpose of this research is to use real-time, quantitative polymerase chain reaction (q-PCR) to quantify cell-free mitochondrial DNA (CF-mtDNA) from the peripheral blood plasma of Mexican-Americans with AD or MCI, as well as non-Hispanic Caucasians with AD or MCI enrolled in the Texas Alzheimer's Research and Care Consortium (TARCC) biobank. The goal is to compare CF-mtDNA between the population subsets. This study was conducted in the hope that it can be useful in future research to assist in identifying the population-specific indices for identification of those

members most at-risk for developing AD based upon CF-mtDNA results (in combination with other biomarkers of disease progression). AD has one of the largest incidences in the Mexican-American population[35], and the disease is generally not diagnosed until the symptoms are irreversible. Most published studies that quantify mtDNA in the context of Alzheimer's disease and cognition utilize Cerebrospinal Fluid (CSF)[39] or brain tissue[1]. These methods are reliable but extremely invasive and, therefore, not ideal for early diagnosis testing. This study explores the efficacy of testing cell-free mtDNA from the peripheral blood plasma as an alternative tissue/indicator of cognitive decline. We hypothesized that CF-mtDNA should have the greatest increase compared to normal controls in AD samples and a slight increase in MCI samples.

Chapter 2: Materials and Methods

Subject selection and characterization

The Tissue Bank and Data Coordinating Center are located at the University of Texas Southwestern Medical Center in Dallas, TX. Samples are provided based on investigator needs and TARCC funded research goals. Samples to be tested include a total cohort (Mexican American and non-Hispanic Caucasian) of 213 individuals. Individuals are diagnosed as AD, MCI, or NC according to the criteria set forth by NINCDS-ADRDA[40 41]. MCI and AD scores are defined through standardized questions of the Clinical Dementia Rating sum of boxes score (CDR SOB)[42 43] Mini-Mental State Examination (MMSE)[40 44] provided through TARCC, among other neurocognitive tests and interviews/questionnaires. Table 1 below describes the total composition of the cohort included in this study.

Table 1: Total Cohort Baseline Characteristics

	AD (N=45)	MCI (N=74)	Control (N=58)	P-value
Gender (male)	53.3%	50%	51.1%	0.939
Age	73.2 (6.0) 58-85	70.5 (6.9) 57-86	70.5 (7.2) 58-86	0.466
Education	11.6 (5.3) 0–20	10.0 (5.4) 0–20	15.4 (2.8) 4–20	0.001
MMSE	21.1 (5.4) 8-30	25.5 (3.4) 16-30	29.3 (0.9) 27-30	<0.001
CDRSOB	5.4 (3.0) 0.5-15	1.05 (6.9) 0-3.0	0.0 (0.01) 0-0.5	<0.001
BMI	27.5 (4.6) 18.1-42.4	29.9 (6.9) 0-51.9	26.5 (6.3) 0-38.1	0.727
Hypertension	48.9%	54.1%	54.3%	0.817

Mean (Standard Deviation) / Range

Table 2: Mexican American Subset Baseline Characteristics

	AD (N=21)	MCI (N=53)	Control (N=18)	P-value
Gender (male)	38.1%	52.8%	61.1%	0.332
Age	75.5 (6.86) 62-85	70.4 (7.8) 57-86	67.9 (5.7) 60-79	0.458
Education	8.1 (5.3) 0–18	8.3 (5.3) 0–20	13.6 (3.0) 4-18	0.008
MMSE	18.5 (5.4) 9-28	24.8 (3.4) 16-30	29.3 (0.8) 27-30	<0.001
CDRSOB	6.3 (2.8) 1-12	1.0 (0.6) 0-3	0.0 (0.1) 0-0.5	<0.001
BMI	26.9 (3.4) 18.1-32.9	30.1 (6.1) 21.9-51.9	30.2 (5.5) 20.2-38.1	0.680
Hypertension	42.9%	60.4%	55.6%	0.393

Mean (Standard Deviation) / Range

Quantifying CF-mtDNA

Circulating Cell-Free mtDNA was extracted from peripheral blood plasma using the Mag-Bind® Blood & Tissue DNA HDQ 96 Kit Omega HDQ kit (Omega Bio-tek) and stored at -80C until used for quantification. The QRL8 assay[45] previously validated in the Phillips lab was used for quantification. CF-mtDNA was quantified using a Taq-Man q-PCR assay on a 7500 Real-Time PCR instrument (Applied Biosystems). This quantification assay is comprised of two amplifications per sample. The first amplification is a target for the NADH dehydrogenase subunit 5 gene. This target requires two HPLC purified primers as well as one TaqMan MGB Probe with a 6FAM reporter dye and a non-fluorescent quencher (NFQ). The second amplification is an internal positive control (IPC). This IPC contains a synthetic template, not found in nature, and is used as a quality control for each sample tested. The IPC requires two primers and one TaqMan probe containing the reporter dye VIC and the quencher TAMRA[45]. The primers used in this project attach to sites 13,288 to 13,392 of the Cambridge Reference Sequence. PCR Amplification Master Mix was prepared under sterile conditions. Each plate was run with a mtDNA standard dilution series of 6 known quantities to create a standard curve. The accuracy of the standard curve was evaluated based on y-intercept, R^2 (>0.995), and the efficiency (>89%). Mean C_T results from the duplicate samples were used to ensure reproducibility for samples with low standard deviation (SD).

Data Analysis

Metadata provided by TARCC was used to determine participant ethnicity (non-Hispanic Caucasian and Mexican American), diagnosis (AD, Mild Cognitive Impairment (MCI), or normal control (NC)), age, gender, and a variety of cognitive (e.g., MMSE, CDR-SOB) and metabolic

phenotypes (e.g., BMI, hypertension). Once labelled, using IBM© SPSS™ Statistics 26 (IBM Corporation, Armonk, NY), the possibility of batch effects was calculated. Plate 1 was identified as an outlier and was not included in analyses presented here. Also, quantification results were tested for normality; the average C_T was transformed using Log 10 to enable parametric testing. Data were entered into the SPSS, and Tables 1 and 2 were generated to compare and contrast the total composition of the cohort against the composition of the Mexican American only portion of the cohort. Independent sampling t-tests and one-way ANOVAs were run to test for group differences between sex, diagnosis, (NC/MCI/AD), cognitive impairment (CI, yes/no, where MCI and AD together into the "yes" group), and hypertension (yes/no); bivariate correlations were generated between the Log10 mean CT between age, BMI, education, CDR-SOB, and MMSE. Analyses were conducted in three stratifications: all participants together, non-Hispanic Caucasian only, and Mexican Americans only). A one-way ANOVA was also run with Log10 CT mean against the diagnosis (AD, MCI, and NC). For analysis of cognitive function, only those individuals with CI were included in the correlation assessments (Log10 CT mean against MMSE and CDRSOB).

Chapter 3: Results

Cohort characteristics.

Cohort characteristics can be found in Tables 1 and 2 above. Samples tested included a total cohort (Mexican American and Caucasian) of 177 individuals (AD=45, MCI=74, NC=58). The Mexican American subset contained 92 of those individuals (AD=21, MCI=53, and NC=18). There were no significant differences in age across the three groups (AD, MCI, and NC).

Correlations with CF-mtDNA.

When analyzing the entire cohort (Table 3), inclusive of both Mexican Americans and non-Hispanic Caucasians, a Pearson correlation coefficient was calculated to weigh the relationship between CF-mtDNA (Log10 mean CT) and age. There was a negative correlation between the two variables, r=-0.198, n= 177, p= 0.008. Figure 2-A summarizes the results. As age increases, the load of CF-mtDNA in plasma decreases. A Pearson correlation coefficient was also calculated for CF-mtDNA and education. There was a negative correlation between the two variables, r=-0.332, n=177, p= >0.001 (Figure 2-B). As there is less education, the higher the content of CF-mtDNA (Figure 2-B). A negative correlation was also found between BMI and education r=-0.224, n= 177, p= 0.003 (Figure 2-C). As BMI increases education decreases. Lastly, a negative correlation was determined between age and BMI r=-0.293, n=177, p= >0.001. In other words, as age increases BMI decreases (Figure 2-D).

Correlations of Total Cohort

		LogCtmean	age	education	BMI
LogCtmean	Pearson Correlation	1	-.198**	-.332**	.143
	Sig. (2-tailed)		.008	.000	.057
	N	177	177	177	177
age	Pearson Correlation	-.198**	1	.064	-.293**
	Sig. (2-tailed)	.008		.398	.000
	N	177	177	177	177
education	Pearson Correlation	-.332**	.064	1	-.224**
	Sig. (2-tailed)	.000	.398		.003
	N	177	177	177	177
BMI	Pearson Correlation	.143	-.293**	-.224**	1
	Sig. (2-tailed)	.057	.000	.003	
	N	177	177	177	177

Table 3: Bivariate correlations of the total cohort with LogCtmean, age, education, and BMI.

**. Correlation is significant at the 0.01 level (2-tailed).

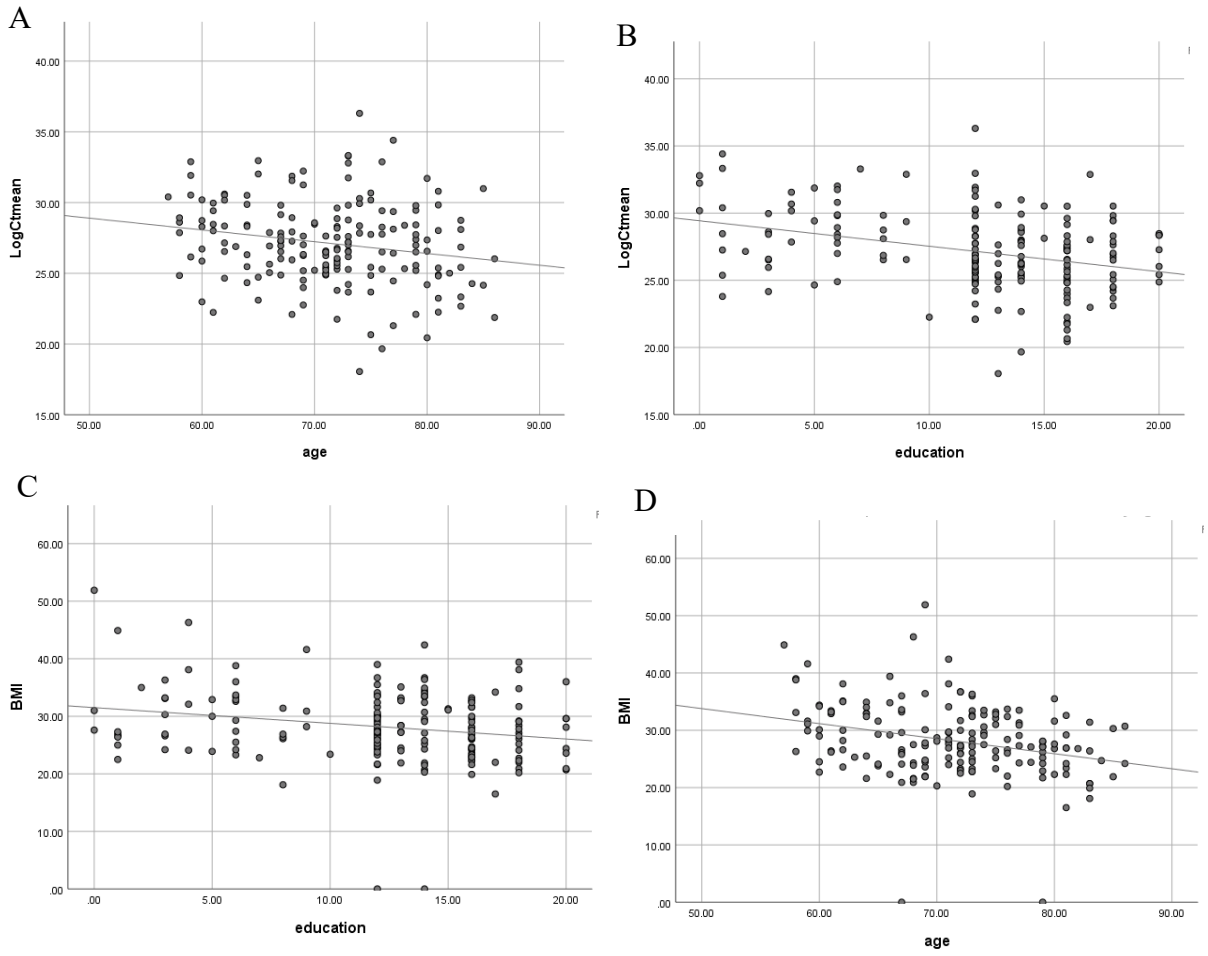

Figure 2: Correlation of significant associations within the total cohort p <0.05. A. CF-mtDNA (Log 10 mean Ct) correlated with age; r=-0.198, p= 0.008. B. CF-mtDNA (Log 10 mean Ct) correlated with education; r=-0.332, p= >0.001; C. BMI correlated with education; r=-0.224, p= 0.003; D. BMI correlated with age; r=-0.293, p= >0.001.

When reviewing the Mexican American subset of the cohort (in Table 4), a Pearson correlation coefficient was calculated to weigh the relationship between BMI and age. There was a negative correlation between the two variables, r=-0.335, n=92, p= 0.001; that is, as age increases, BMI decreases. Figure 3-A summarizes the results. A Pearson correlation coefficient was also calculated for education and CF-mtDNA. There was a negative correlation between the two variables, r=-0.334, n=92, p= 0.001. As CF-mtDNA increases education decreases (Figure 3-B).

Correlations of Mexican American Subset

		LogCtmean	age	education	BMI
LogCtmean	Pearson Correlation	1	-.148	-.344**	.145
	Sig. (2-tailed)		.159	.001	.167
	N	92	92	92	92
age	Pearson Correlation	-.148	1	.081	-.335**
	Sig. (2-tailed)	.159		.441	.001
	N	92	92	92	92
education	Pearson Correlation	-.344**	.081	1	-.147
	Sig. (2-tailed)	.001	.441		.162
	N	92	92	92	92
BMI	Pearson Correlation	.145	-.335**	-.147	1
	Sig. (2-tailed)	.167	.001	.162	
	N	92	92	92	92

Table 4: Bivariate correlations of the Mexican American subset with LogCtmean, age, education, and BMI.

**. Correlation is significant at the 0.01 level (2-tailed).

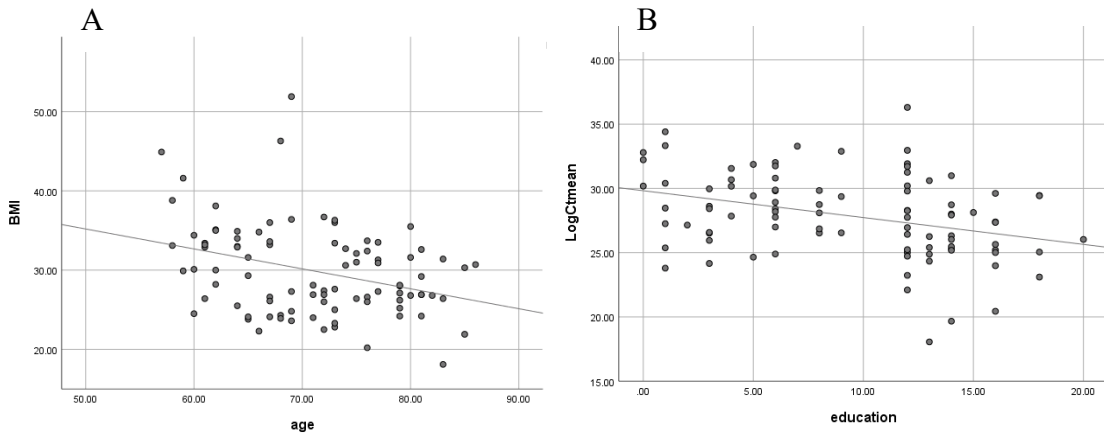

Figure 3: Correlation of significant associations within Mexican American subset p <0.05. A. BMI correlated with age; r=-0.335, p= 0.001. B. CF-mtDNA (Log 10 mean Ct) correlated with education; r=-0.334, p= 0.001.

When studying the non-Hispanic Caucasian subset of the cohort (Table 5), a Pearson correlation coefficient was calculated to weigh the relationship between CF-mtDNA (Log10 mean CT) and age. There was a negative correlation between the two variables, r=-0.280, n=85, p= 0.009; that is, as age increases, the load of CF-mtDNA in plasma decreases as shown in Figure 4-A. A Pearson

correlation coefficient was also calculated for age and BMI. There was a negative correlation between the two variables, r=-0.256, n=85, p= 0.018. As age increases BMI decreases (Figure 4-B).

Correlations of non-Hispanic Caucasian Subset

		LogCtmean	age	education	BMI
LogCtmean	Pearson Correlation	1	-.280**	.111	-.015
	Sig. (2-tailed)		.009	.314	.893
	N	85	85	85	85
age	Pearson Correlation	-.280**	1	-.020	-.256*
	Sig. (2-tailed)	.009		.856	.018
	N	85	85	85	85
education	Pearson Correlation	.111	-.020	1	.031
	Sig. (2-tailed)	.314	.856		.777
	N	85	85	85	85
BMI	Pearson Correlation	-.015	-.256*	.031	1
	Sig. (2-tailed)	.893	.018	.777	
	N	85	85	85	85

Table 5: Bivariate correlations of the non-Hispanic Caucasian subset with LogCtmean, age, education, and BMI

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

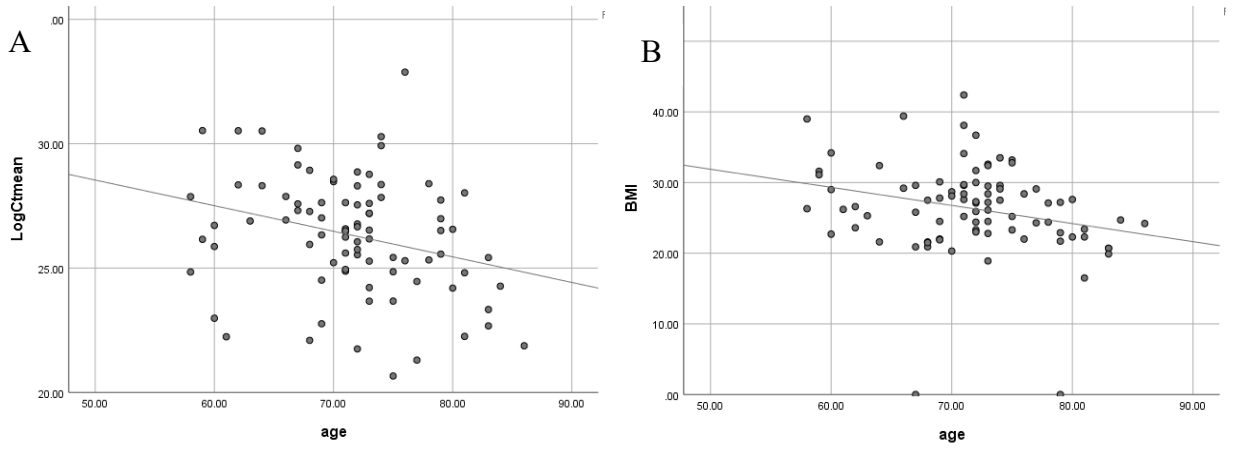

Figure 4: Non-Hispanic Caucasian subset only with all diagnoses $p < 0.05$. A. CF-mtDNA (Log 10 mean Ct) correlated with age; r=-0.280, p= 0.009. B. BMI correlated with age; r=-0.256, p= 0.018.

When analyzing the entire cohort (Table 6), inclusive of both Mexican Americans and non-Hispanic Caucasians, with Cognitive Impairment only, a Pearson correlation coefficient was calculated to weigh the relationship between CF-mtDNA (Log10 mean CT) and MMSE. There was a negative correlation between the two variables, r=-0.210, n=119, p= 0.022; that is, as CF-mtDNA increases, the MMSE score decreases as shown in Figure 5-A. A Pearson correlation coefficient was also calculated for CDRSOB and MMSE. There was a negative correlation between the two variables, r=-0.692, n=119, p= >0.001; that is, as the CDRSOB score decreases the higher the MMSE score (Figure 5-B).

Correlations of Total Cohort with CI

		MMSE	CDRSOB	LogCtmean
MMSE	Pearson Correlation	1	-.692**	-.210*
	Sig. (2-tailed)		.000	.022
	N	119	119	119
CDRSOB	Pearson Correlation	-.692**	1	.063
	Sig. (2-tailed)	.000		.499
	N	119	119	119
LogCtmean	Pearson Correlation	-.210*	.063	1
	Sig. (2-tailed)	.022	.499	
	N	119	119	119

Table 6: Bivariate correlations of the total cohort with CI (no controls) with LogCtmean, age, education, and BMI.

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

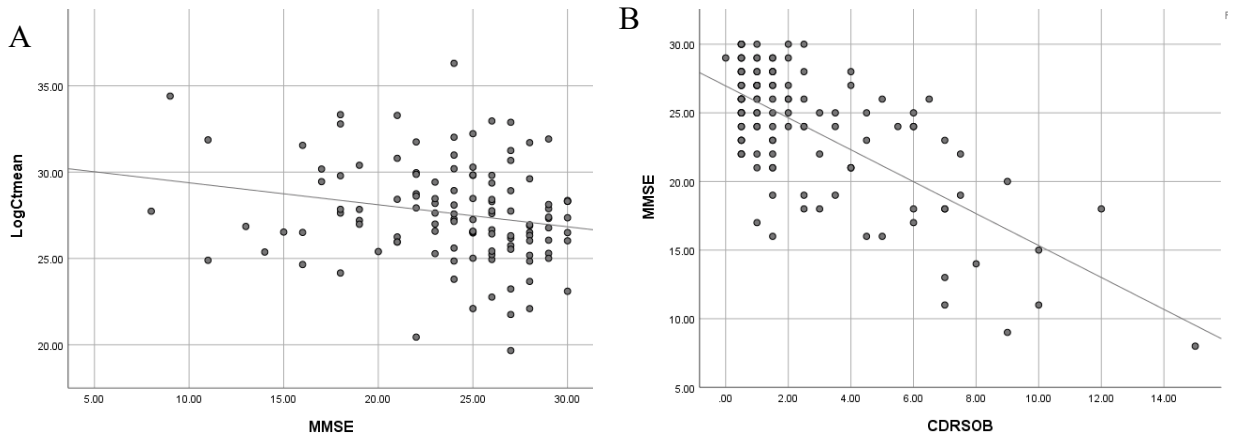

Figure 5: Total cohort with only CI p <0.05. A. CF-mtDNA (Log 10 mean Ct) correlated with MMSE; r=-0.210 p= 0.022. B. MMSE correlated with CDRSOB; r=-0.692, p= >0.001.

When exploring the Mexican American cohort with Cognitive Impairment only (Table 7), a Pearson correlation coefficient was calculated for CDRSOB and MMSE. There was a negative correlation between the two variables, as would be fully expected, r=-0.697, n=74, p= >0.001; that is, as the MMSE score increases, the CDRSOB score decreases (Figure 6).

When evaluating the non-Hispanic Caucasian cohort, with Cognitive Impairment only (Table 8), a Pearson correlation coefficient was calculated to weigh the relationship between CF-mtDNA (Log10 mean CT) and CDRSOB. There was a positive correlation between the two variables, r=0.369, n=45, p= 0.013; that is, as CF-mtDNA increases, the CDRSOB score also increases as shown in Figure 7-A. A Pearson correlation coefficient was also calculated for CDRSOB and MMSE. There was a negative correlation, as is fully expected, between the two variables, r=-0.808, n=45, p= >0.001; that is, as the CDRSOB score decreases the higher the MMSE score (Figure 7-B).

Correlations of Mexican American Subset with CI

		MMSE	CDRSOB	LogCtmean
MMSE	Pearson Correlation	1	-.697**	-.144
	Sig. (2-tailed)		.000	.221
	N	74	74	74
CDRSOB	Pearson Correlation	-.697**	1	.004
	Sig. (2-tailed)	.000		.973
	N	74	74	74
LogCtmean	Pearson Correlation	-.144	.004	1
	Sig. (2-tailed)	.221	.973	
	N	74	74	74

Table 7: Bivariate correlations of the Mexican American subset with CI (no controls) with LogCtmean, age, education, and BMI.

**. Correlation is significant at the 0.01 level (2-tailed).

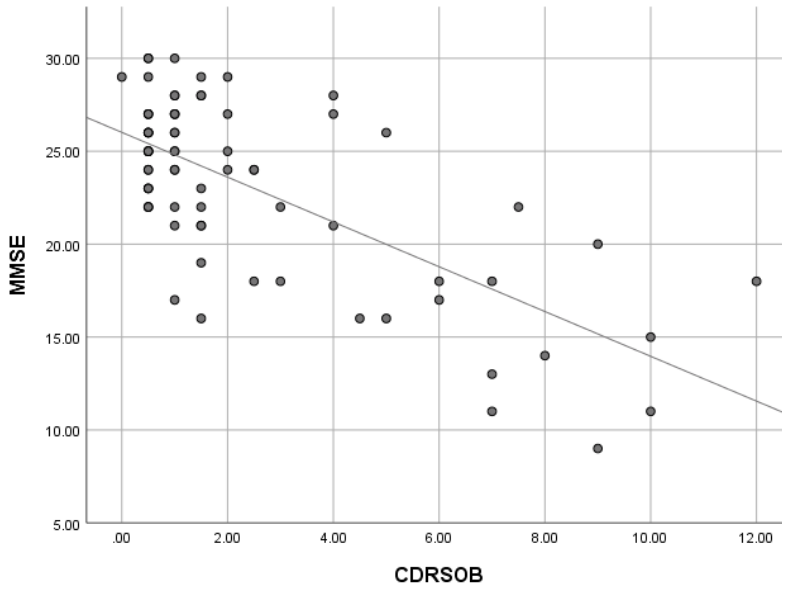

Figure 6: Mexican American subset with only CI p <0.05. MMSE correlated with CDRSOB; r=-0.697, p= >0.001.

Correlations of non-Hispanic Caucasian Subset with CI

		MMSE	CDRSOB	LogCtmean
MMSE	Pearson Correlation	1	-.808**	-.180
	Sig. (2-tailed)		.000	.238
	N	45	45	45
CDRSOB	Pearson Correlation	-.808**	1	.369*
	Sig. (2-tailed)	.000		.013
	N	45	45	45
LogCtmean	Pearson Correlation	-.180	.369*	1
	Sig. (2-tailed)	.238	.013	
	N	45	45	45

Table 8: Bivariate correlations of the non-Hispanic Caucasian subset with CI (no controls) with LogCtmean, age, education, and BMI.

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

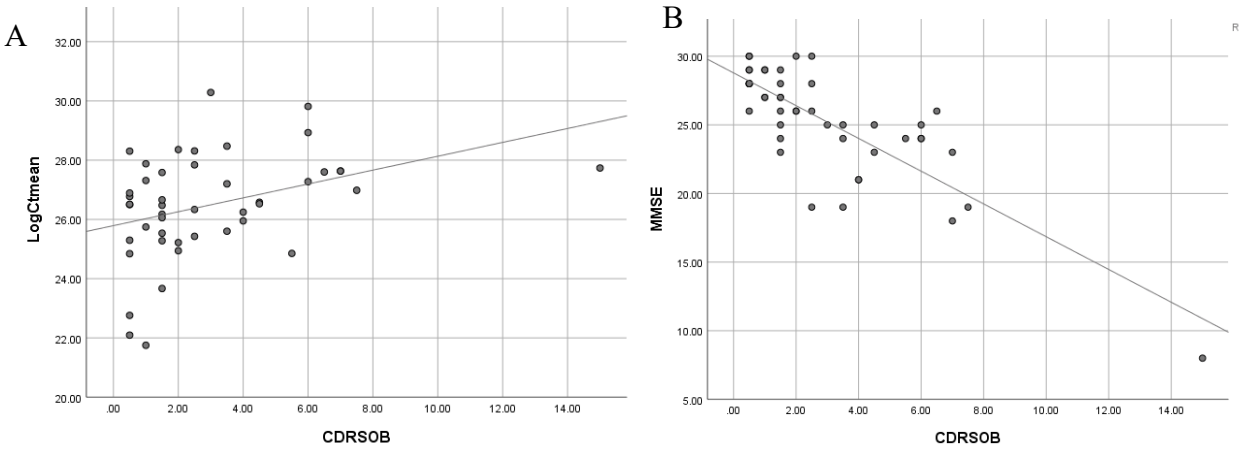

Figure 7: Non-Hispanic Caucasian subset with only CI p <0.05. A. CF-mtDNA (Log 10 mean Ct) correlated with CDRSOB; r=0.369, p= 0.013. B. MMSE correlated with CDRSOB; r=-0.808, p= >0.001.

Group differences in CF-mtDNA.

A one-way ANOVA was conducted to compare the effect of diagnosis on CF-mtDNA quantities in AD, MCI, and NC samples in each subset (total, Mexican American, non-Hispanic Caucasian). There was a significant effect of diagnosis on CF-mtDNA at the $p<0.05$ level for the three conditions in the total cohort [$F(2,174) = 5.9$, $p=0.003$]. Post hoc comparisons using Tukey HSD test reported a significant difference in the MCI and NC diagnoses in the total cohort ($p=0.002$). However, the AD and NC diagnoses did not significantly differ in the total cohort. In the Mexican American subset, there was a significant effect of diagnosis on CF-mtDNA at the $p<0.05$ level for the three conditions in the total cohort [$F(2,89) = 4.2$, $p=0.019$]. Post hoc comparisons using Tukey HSD test reported a significant difference in the MCI and NC diagnoses in the total cohort ($p=0.014$). Though, the AD and NC diagnoses did not significantly differ in the Mexican American subset. In the non-Hispanic Caucasian subset, there was not a significant effect of diagnosis on CF-mtDNA at the $p<0.05$ level for the three conditions in the total cohort [$F(2,82) = 1.1$, $p=0.351$]. Taken together, these results suggest that there is a significant change in CF-mtDNA quantities from NC to MCI individuals (Figure 8).

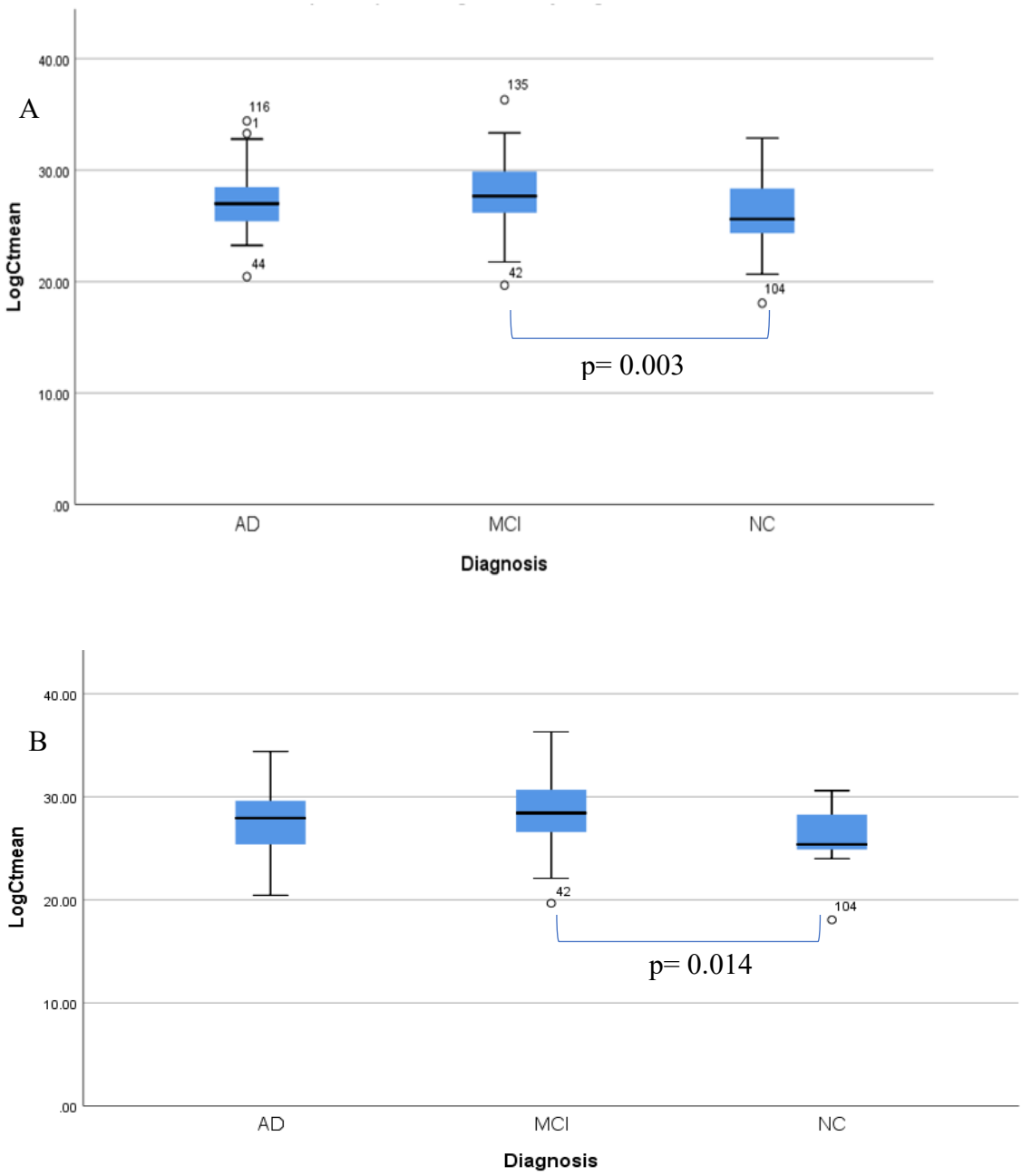

Figure 8: Comparison of the effect of diagnosis on CF-mtDNA quantities in AD, MCI, and NC samples in the A. total cohort B. Mexican American subset.

An independent-samples t-test was conducted to compare CF-mtDNA in individuals with and without CI in each subset (total, Mexican American, and non-Hispanic Caucasian). There was a significant difference in the scores for CF-mtDNA in individuals with CI (M= 27.6, SD=2.88) and without CI (M=26.1, SD=2.87) in the total cohort; t(175)=3.28, p=0.001. There was a significant

difference in the scores for CF-mtDNA in individuals with CI (M= 28.3, SD=3.2) and without CI (M=26.1, SD=2.9) in the Mexican American subset; t(90)=2.7, p=0.008. There was not a significant difference in the scores for CF-mtDNA in individuals with CI (M= 26.5, SD=1.8) and without CI (M=26.1, SD=2.9) in the non-Hispanic Caucasian subset; t(83)=0.701, p=0.485. These results suggest CI has an effect on CF-mtDNA quantities only in the Mexican American subset.

An independent-samples t-test was conducted to compare CF-mtDNA in male and female individuals in each subset (total, Mexican American, and non-Hispanic Caucasian). There was a significant difference in the scores for CF-mtDNA in male individuals (M=26.6, SD=2.67) and female individuals (M=27.7, SD=3.2) in the total cohort; t (175) =-2.4, p=0.017 (Figure 10-A). There was a significant difference in the scores for CF-mtDNA in male individuals (M=27.1, SD=3.0) and female individuals (M=28.7, SD=3.4) in the Mexican American subset; t (90) =-2.4, p=0.020(Figure 10-B). There was not a significant difference in the scores for CF-mtDNA in male individuals (M=26.1, SD=2.2) and female individuals (M=26.6, SD=2.5) in the non-Hispanic Caucasian subset; t(83)=-0.936, p=0.352. These results suggest that sex has an effect on CF-mtDNA quantities only in the Mexican American subset.

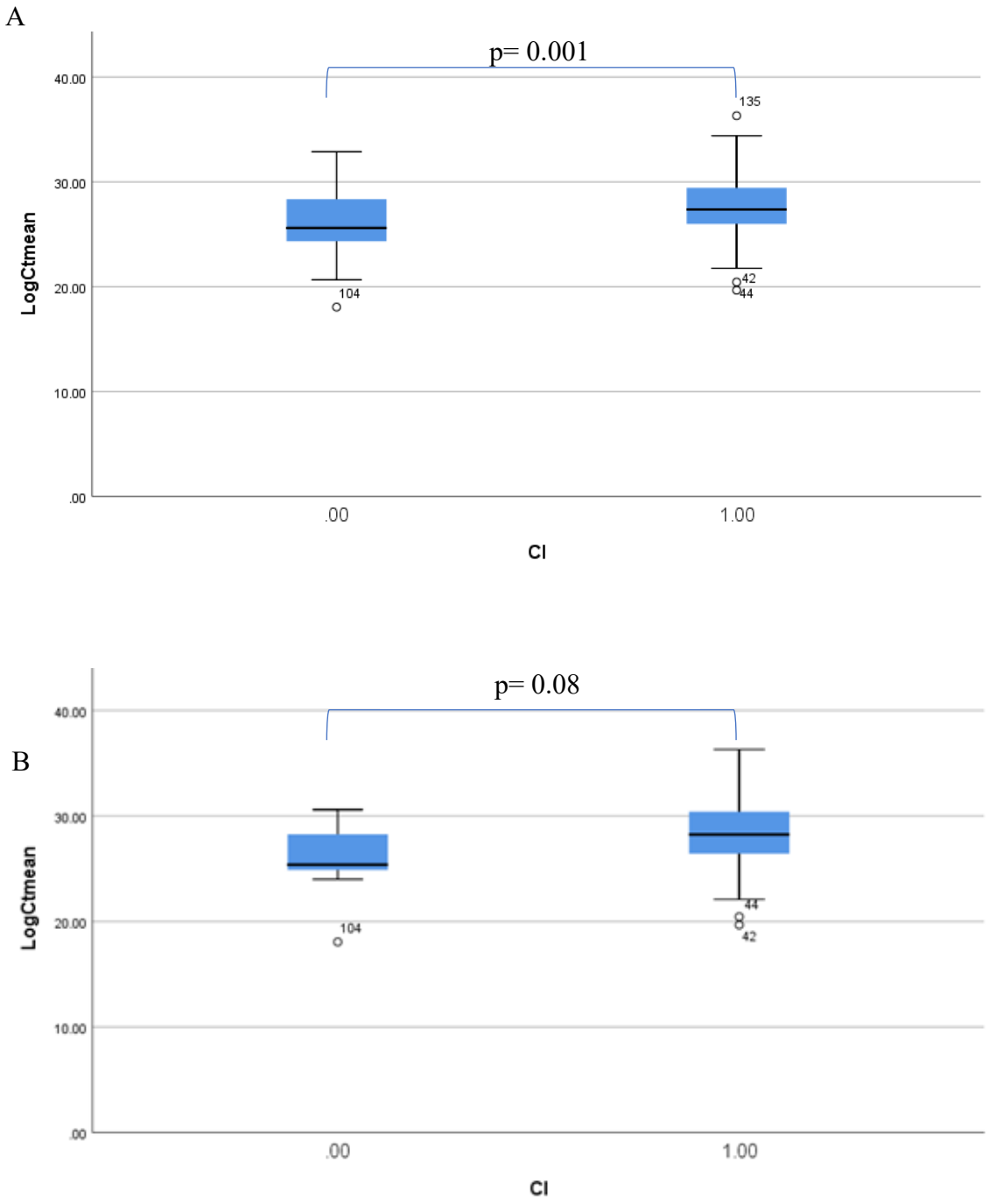

Figure 9: Comparison of the effect of CI on CF-mtDNA quantities in samples with and without CI in the A. total cohort B. Mexican American subset. No CI=0; CI=1.

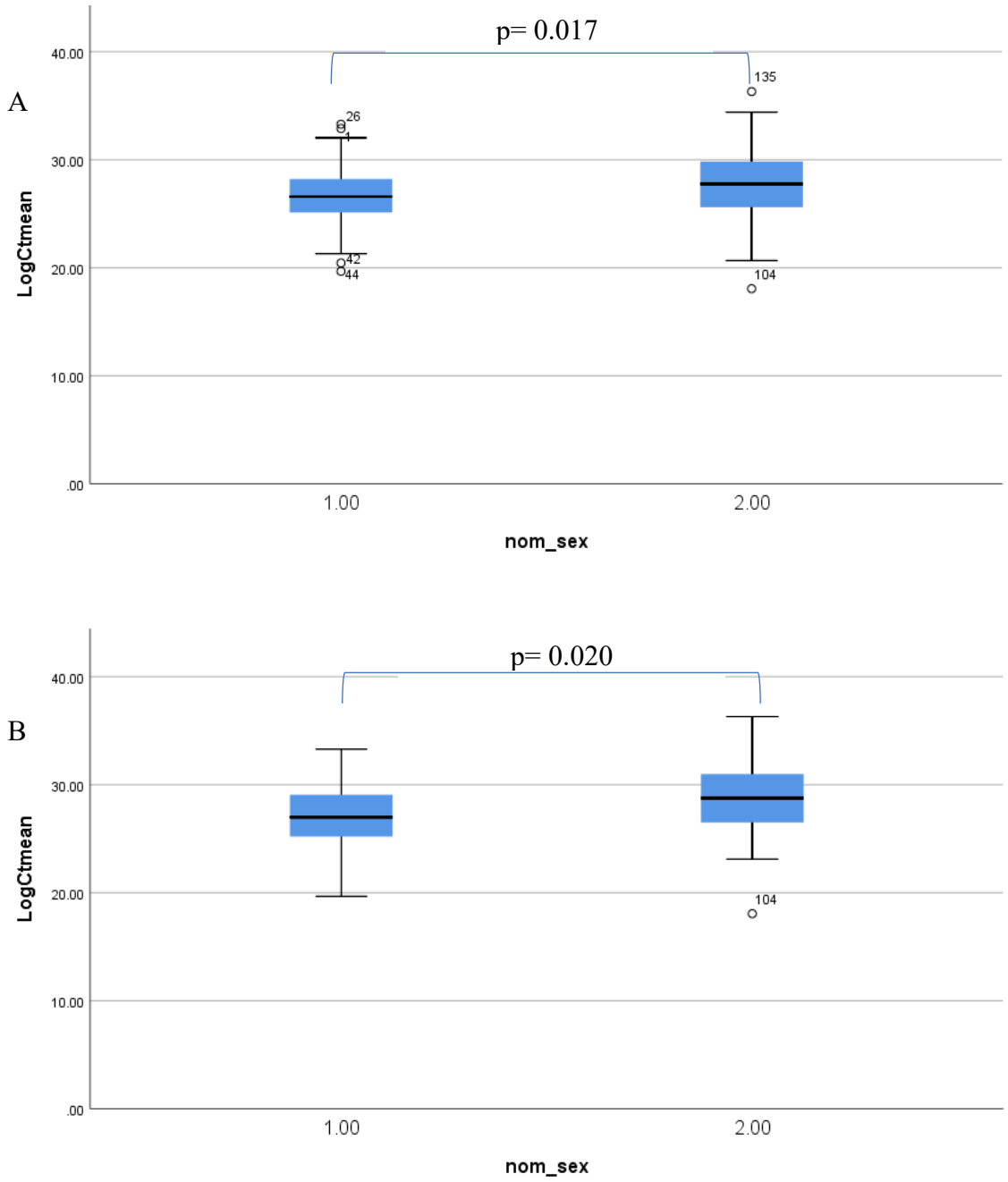

Figure 10: Comparison of the effect of CI on CF-mtDNA quantities in samples with and without CI in the A. total cohort B. Mexican American subset. Male=1; Female=2.

An independent-samples t-test was conducted to compare CF-mtDNA in individuals with and without hypertension in each subset (total, Mexican American, and non-Hispanic Caucasian).

There was not a significant difference in the scores for CF-mtDNA in individuals with hypertension (M=26.9, SD=3.1) and without hypertension (M=27.4, SD=2.8) in the total cohort; t(175)=-0.991, p=0.323. There was not a significant difference in the scores for CF-mtDNA in individuals with hypertension (M= 27.9, SD=3.4) and without hypertension (M=27.8, SD=3.1) in the Mexican American subset; t(90)=0.14, p=0.889. There was a significant difference in the scores for CF-mtDNA in individuals with hypertension (M= 25.7, SD=2.1) and without hypertension (M=26.9, SD=2.4) in the non-Hispanic Caucasian subset; t(83)=-2.5, p=0.014. These results suggest that hypertension has an effect on CF-mtDNA quantities. Specifically, in the non-Hispanic Caucasian subset which showed the only significant difference.

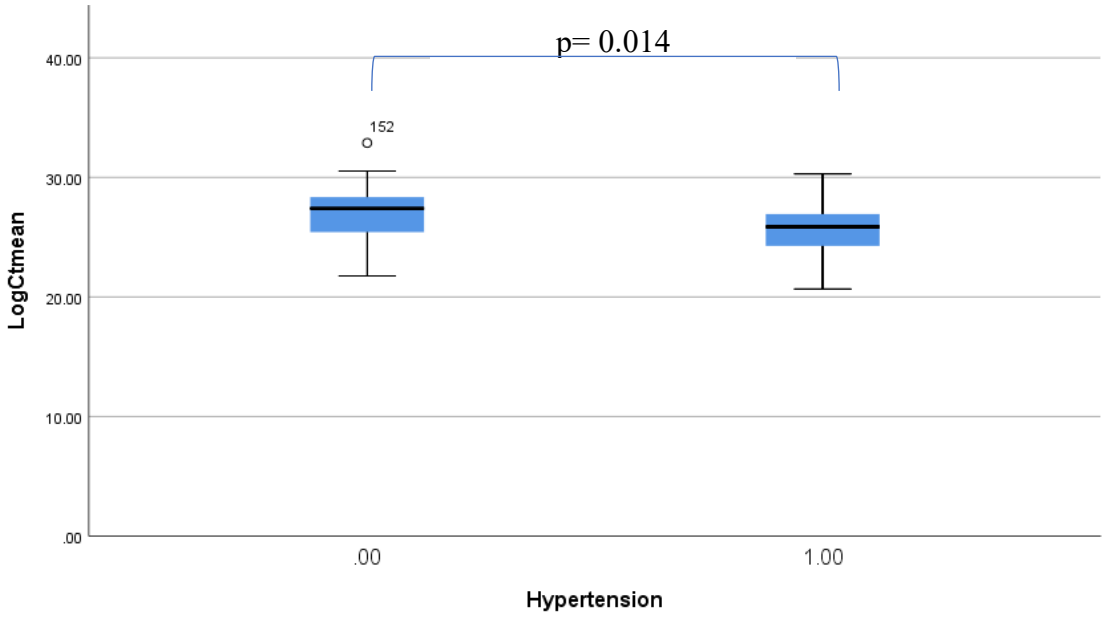

Figure 11: Comparison of the effect of hypertension on CF-mtDNA quantities in samples with and without hypertension in the non-Hispanic Caucasian subset. No hypertension=0; Hypertension=1.

A one-way ANOVA was conducted to compare the effect of diagnosis on CF-mtDNA quantities in AD, MCI, and NC samples in the males and female populations (from the total cohort). There was not a significant effect of diagnosis on CF-mtDNA at the $p<0.05$ level for the three conditions in the male population [$F(2,88) = 2.8$, $p=0.064$]. In the female population, there was, similarly, not a significant effect of diagnosis on CF-mtDNA at the $p<0.05$ level for the three conditions in the total cohort [$F(2,83) = 3.0$, $p=0.053$]. These results indicate that sex does not have an overall significant effect on CF-mtDNA quantities.

Chapter 4: Discussion

The Mexican American subset and combined total cohort had a few differences that we should be aware of when analyzing the results. For example, the Mexican American subset was slightly more overweight, more hypertensive, less educated, older, and had more impaired cognition scores than the total cohort. Both groups had roughly the same amount of men and women therefore sex had reduced bias in this study.

An increase of CF-mtDNA circulating in the bloodstream may indicate an issue with mitochondrial quality control and therefore, mitochondrial dysfunction. As stated previously, CF-mtDNA is now being used as a biomarker incredibly early on in the diagnosis of AD. Due to this, we expected to see the greatest difference in CF-mtDNA quantity in NC to AD samples. Instead, the most significant difference was between MCI and NC samples. The measurements for Log 10 mean Ct and MMSE and/or CDRSOB were found to be negatively correlated. In the case of MMSE scores, the lower the score the more impaired the individual. In CDRSOB scores, it is the opposite. The higher the score the more impaired the individual. Therefore, as CF-mtDNA quantity increased, the MMSE and CDRSOB scores were less impaired. This was a thought-provoking result. The

hypothesis stated that we would find the highest levels of CF-mtDNA in the more impaired individuals, but the results conclude that the highest levels of CF-mtDNA are in the less impaired individuals. Although these results are certainly contradictory to our expectations, it is worth noting that our analysis does not characterize the nature of the mtDNA in circulation—i.e., is the DNA damaged or oxidized? Are the fragments large or small? How are they packaged—in exosomes or perhaps within intact mitochondrial organelles? All of these variables have shown[46-48] to influence the way that mtDNA propagates signaling and are a subject of future research.

In this study, we tested for many factors that could influence CF-mtDNA quantities in the bloodstream and any one of these (i.e. sex, age, education) could be a potentially confounding factor for MMSE and CDRSOB scores. Since these results do not take in to account any variables, this likely explains the counter-intuitive result. In addition to correlation analyses, logistic and linear regression models should be used as a supplement to assess the effect of CF-mtDNA in the absence of potentially confounding covariates.

The Mexican American and non-Hispanic Caucasian subset showed differences in correlations, indicating that the two subsets are not demonstrating the same effects. However, they are in the presence of confounding variables and this could explain the phenomenon analyzed. The only correlation that is consistent within all subset variants (total, Mexican American, and non-Hispanic Caucasian) is with age. This suggests our data is most likely correct, but we lack power to detect the group effects for cognitive phenotypes. In this study, the cognitive phenotype effects seen in correlations are confounded by the blinded sample selection for the tests conducted.

Limitations

Though the results of this study should be considered reliable, there are a few limitations that should be acknowledged. Sample size is the biggest limitation. Our study is limited to participants from a local, Texas specific, biobank. Future studies would expand the cohort to multiple biobanks and/or databases to allow for a more robust result. During the analysis of the metadata, it was discovered that that three groups were disproportionately represented on each of the plates. Plate 1, which was ultimately removed from calculations, and Plate 4 contained only normal controls. Two other plates did not contain any NC samples, and the other 3 had even distribution of all three groups. While it is possible this distribution of conditions may skew the data one way or another, every precaution was taken during the data analysis step to avoid batch bias. Also, participants were self-reporting medical history so there is a potential for underrepresenting the diseases we are interested in within this cohort. Second, though our mtDNA was obtained through peripheral blood plasma, and therefore contains an abundant amount of mtDNA, the possibility of nuclear DNA contamination is a possibility. To combat this, we propose to continue this research using a duplex quantification assay to account for nuclear DNA as well as mtDNA copy numbers in every sample tested.

Chapter 5: Conclusion

In conclusion, our analysis of this sub-set of samples for the TARCC study highlights the need for additional power and analysis methods which are able to account for confounding factors when analyzing the complete TARCC dataset for this project; CF-mtDNA is an easily accessible and easily tested molecular marker of diseases that are relevant to studies for cognitive decline.

Although our findings in the preliminary correlation and group comparison tests were inconsistent with current literature, they bring to light the weight of confounding factors within limited sample studies. In future studies, a more robust sample set should be used alongside accounting for more potential covariates.

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