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Research paper

Platelet cytochrome oxidase and citrate synthase activities in APOE ε4 carrier and non-carrier Alzheimer's disease patients

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ABSTRACT

A degradation product of *APOE ε4*-encoded apolipoprotein E protein targets mitochondria and inhibits cytochrome oxidase (COX), and autopsy brains from young adult *APOE ε4* carriers show reduced COX activity. To further explore relationships between *APOE* alleles and COX, we measured platelet mitochondria COX activity in AD subjects with (n = 8) and without (n = 7) an *APOE ε4* allele and found the mean COX activity, when normalized to sample total protein, was lower in the *APOE ε4* carriers ($p < 0.05$). Normalizing COX activity to citrate synthase (CS) activity eliminated this difference, but notably the mean CS activity was itself lower in the *APOE ε4* carriers ($p < 0.05$). COX and CS protein levels did not appear to cause the lower *APOE ε4* carrier COX and CS Vmax activities. If confirmed in larger studies, these data could suggest mitochondria at least partly mediate the well-recognized association between *APOE* alleles and AD risk.

1. Introduction

Apolipoprotein E (APOE) variants associate with Alzheimer's disease (AD), with the *APOE* ε2, ε3, and ε4 alleles respectively defining reduced, intermediate, and increased risk [1–6]. Different mechanisms may mediate this, including mechanisms that invoke mitochondrial function [1,7–12]. Relevant to this possibility, *APOE*-mitochondria relationships occur outside the context of an AD diagnosis. For instance, Valla et al. reported autopsy brains from young adult *APOE ε4* carriers show reduced cytochrome oxidase (COX) activity, at a point that predates differences in beta amyloid protein [13,14]. Fluorodeoxyglucose positron emission tomography (FDG PET) studies also show reduced glucose utilization in asymptomatic, middle-aged *APOE ε4* carriers, which could reflect mitochondria-related bioenergetic differences [15,16].

From an epidemiologic perspective, two studies report *APOE* genotypes and mitochondrial DNA (mtDNA) haplogroups actually interact to define AD risk [17,18]. This interaction would presumably converge at the level of mitochondrial function. Also, the *TOMM40* gene, which encodes a critical mitochondrial translocase, borders the *APOE* gene and as a result *TOMM40* and *APOE* allele signatures show linkage disequilibrium [2,19–21]. This raises the possibility that *APOE* alleles could relate indirectly to mitochondrial function through *TOMM40* linkage disequilibrium. Even if an indirect association such as this does exist, though, *APOE* genotypes may still directly influence mitochondrial function via the production of apolipoprotein E isoform-dependent degradation products. Specifically, cleavage of the *APOE ε4*-derived apolipoprotein E isoform (but not other isoforms) generates a mitochondrial targeting sequence and this mitochondria-targeted peptide inhibits COX [8,9,22].

A mechanistic link between *APOE ε4* and COX seems particularly intriguing, as AD subject groups show lower mean COX activities than age-matched control groups [23]. The AD COX deficit manifests across several tissues, including platelets [24]. mtDNA contributes to the AD COX activity reduction [25], but this does not rule out the possibility that other modifiers or potential modifiers of mitochondrial function, including *TOMM40* or *APOE*, play a role.

In this study, we measured platelet mitochondria COX activities in *APOE ε4* carrier and non-carrier AD patients. To test whether *APOE* allele types relate to COX activity, we compared the total protein and

Abbreviations: AD, Alzheimer's disease; APOE, apolipoprotein E; COX, cytochrome oxidase; COX4I1, cytochrome oxidase subunit 4 isoform 1; CS, citrate synthase; FDG PET, Fluorodeoxyglucose positron emission tomography;; mtDNA, mitochondrial DNA; SD, standard deviation; SEM, standard error measurement; SNP, single nucleotide polymorphism; TOMM40, translocase of the outer mitochondrial membrane

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citrate synthase (CS)-referenced inter-group COX activity means.

2. Materials and methods

2.1. Approvals and Human Subjects

This study in part includes a secondary analysis of data acquired during the course of the S-equol in Alzheimer's Disease (SEAD) trial (ClinicalTrials.gov NCT02142777; manuscript in review). The Kansas University Medical Center Human Subjects Committee approved the SEAD protocol and all participants provided informed consent prior to enrolling. This study was conducted in accordance with the Code of Ethics of the World Medical Association (the Declaration of Helsinki). We enrolled fifteen female participants (12 Caucasians and 3 African Americans), all met McKhann et al. AD diagnostic criteria [26], and all had clinical dementia rating (CDR) scores of 0.5 or 1 [26]. Participants were exluded if they reported any potentially confounding, serious medical risks such as type 1 diabetes, cancer, or a recent cardiac event such as a heart attack or angioplasty. Each subject had a study partner who was engaged in their daily social and medical care. Over the course of the trial partipants underwent three 40 ml phlebotomies, which occurred at two week intervals, and we determined platelet mitochondria COX and CS activities for each phlebotomy (see below). The first phlebotomy occurred during a placebo lead-in period, the second occurred two weeks after initiating the study drug, and the third occurred after a two-week treatment wash-out period.

2.2. Phlebotomy and mitochondrial isolation

Forty ml of blood was collected in tubes containing acid-citrate-dextrose anticoagulant. One ml of whole blood was removed and stored at −80 °C for genotyping; the rest was used for platelet harvesting and mitochondrial enrichment.

Briefly, 15 ml of Histopaque 1077 was centrifuged in an AccuSpin tube for 1 min at 1700 × *g*. Blood was layered on top of the AccuSpin tube frit, and centrifuged for 15 min at 400 × *g*. Platelet-rich plasma was collected and platelets were pelleted by centrifugation for 15 min at 1700 × *g*. The platelet pellet was washed with phosphate buffered saline (PBS). Platelets were then resuspended in MSHE buffer (225 mM mannitol, 75 mM sucrose, 5 mM Hepes, 1 mM EGTA, pH 7.4) and disrupted by nitrogen cavitation, at 1200 psi, for 20 min. The ruptured platelets were centrifuged at 1000 × *g* for 15 min, 4 °C. The supernatent was transferred to a new tube, while the pellet (intact platelets) was resuspened in MSHE buffer and subjected to nitrogen caviation for a second time (1200 psi for 20 min). Both supernatents were combined and centrifuged at 12,000 × *g* for 10 min, 4 °C. The resulting mitochondrial pellet was resuspended in MSHE buffer.

2.3. COX and CS Vmax Assays

We added aliquots of the enriched mitochondrial suspensions to cuvettes and spectrophotometrically determined each suspension's COX and CS Vmax activities. For the COX Vmax, we followed the conversion of reduced cytochrome c to oxidized cytochrome c, and calculated the pseudo-first order rate constant ($msec^{-1}$). For the CS Vmax, we followed the formation of 5-thio-2-nitrobenzoate (nmol/min) [27]. The COX rate was normalized to mg protein ($msec^{-1}$/mg protein) or to the CS rate (yielding a value with units of $msec^{-1}$/nmol/min), which we herein refer to simply as COX/CS. The CS rate was normalized to mg protein to yield a final activity with units of nmol/min/mg protein.

2.4. Immunochemistry

For mitochondrial suspensions that contained, following the Vmax assays, adequate amounts of protein we used a Western blot approach to measure COX subunit 4 isoform 1 (COX4I1) and CS protein levels. Briefly, equal protein amounts were resolved by SDS-PAGE (Criterion TGX gels, BioRad) and proteins were tranfered to nitrocellulose membranes (ThermoFisher). Immunoblots against COX4I1 (Cell Signaling, 1:500) and CS (Cell Signaling, 1:500) were analyzed by densitometry. To visualize bands, we used WestFemto Super Signal HRP Substrate (ThermoFisher) and a ChemiDoc XRS imaging platform.

2.5. APOE and TOMM40 genotyping

We used a single nucleotide polymorphism (SNP) allelic discrimination assay to determine *APOE* genotypes. This involved adding 5 µl of blood to a Taqman Sample-to-SNP kit (ThermoFisher). Taqman probes to the two *APOE*-defining SNPs, rs429358 (C_3084793_20) and rs7412 (C_904973_10) (ThermoFisher), were used to identify *APOE ε2*, *ε3*, and *ε4* alleles.

Intron 6 of the *TOMM40* gene contains a variable length poly-T region that defines the rs10524523 SNP (referred to herein as *TOMM40 `523)* [21]. To obtain the `523 poly-T length, we used a DNeasy kit (Qiagen) to prepare genomic DNA from 100 µl of whole blood and PCR-amplified the DNA with the following primers: 5′-6FAM-CTGACCTCA-AGCTGTCCTC-3′ (forward) and 5′-GAGGCTGAGAAGGGAGGATT-3′ (reverse). The PCR reaction contained 0.2 µM of each primer, TaKaRa EX Taq polymerase (Takara Bio Inc), and 5% DMSO. The PCR parameters were 1 cycle of 5 min at 94 °C; 30 cycles of 30 s at 94 °C, 30 s at 65 °C, and 30 s at 72 °C; and 1 cycle of 5 min at 72 °C. After using a QIAQuick PCR purification kit to remove nucleotides and primers, we submitted the amplicons to Genewiz, Inc. for fragment analysis. The *TOMM40* poly-T length was calculated by subtracting the size of the PCR product from 150 (the expected size of the PCR product lacking the poly-T region) as previously described [28]. In preliminary experiments, we showed this assay identified the *TOMM40* poly-T length to within +/−1 nucleotides. We used the parameters defined by Roses et al. to place each allele into one of three groups, in which a short (S) allele represents a poly-T length of $\leq$19, a long (L) allele represents a poly-T length of 20–29, and a very long (VL) allele represents a poly-T length of $\geq$30 [19,20,28].

2.6. Data and statistical analyses

Our enzyme activity analysis did not include measurements from platelets obtained during the active S-equol treatment period. Whenever possible we averaged the two enyzme activity values from the pre-treatment lead-in and post-treatment wash-out periods to yield a single final value; 14 of the 15 participants had complete data sets. One participant had only one set of valid CS and COX/CS values, and that participant's CS and COX/CS activities represent single rather than averaged measurements.

We organized the data from the 15 participants into two groups, one in which participants had at least one *APOE ε4* allele, and one in which they did not. We also grouped the participants by their *TOMM40 L* status, to define *TOMM40 L* carrier and non-carrier groups. We summarized group COX (per total protein), CS (per total protein), and COX/CS activities by means, standard deviations (SD), and standard error of the means (SEM) and compared means by two-way Student's T-tests with significance defined as $p < 0.05$.

3. Results

The *APOE ε4* carrier (n = 8; all heterozygous) and non-carrier (n = 7) group mean ± SEM ages were, respectively, 70.9 ± 2.2 and 76.4 ± 3.4 (p = 0.17), and the group CDR ± SEM scores were 0.75 ± 0.09 and 0.86 ± 0.09 (p = 0.43). The *APOE ε4* carrier group COX activity, when normalized to total protein, was 27% lower than that of the *APOE ε4* non-carrier group (p = 0.039). The mean CS activity in the *APOE ε4* carriers was 34% lower than that of the *APOE ε4* non-carriers (p = 0.049). The COX/CS activities were comparable (Fig. 1). Adequate

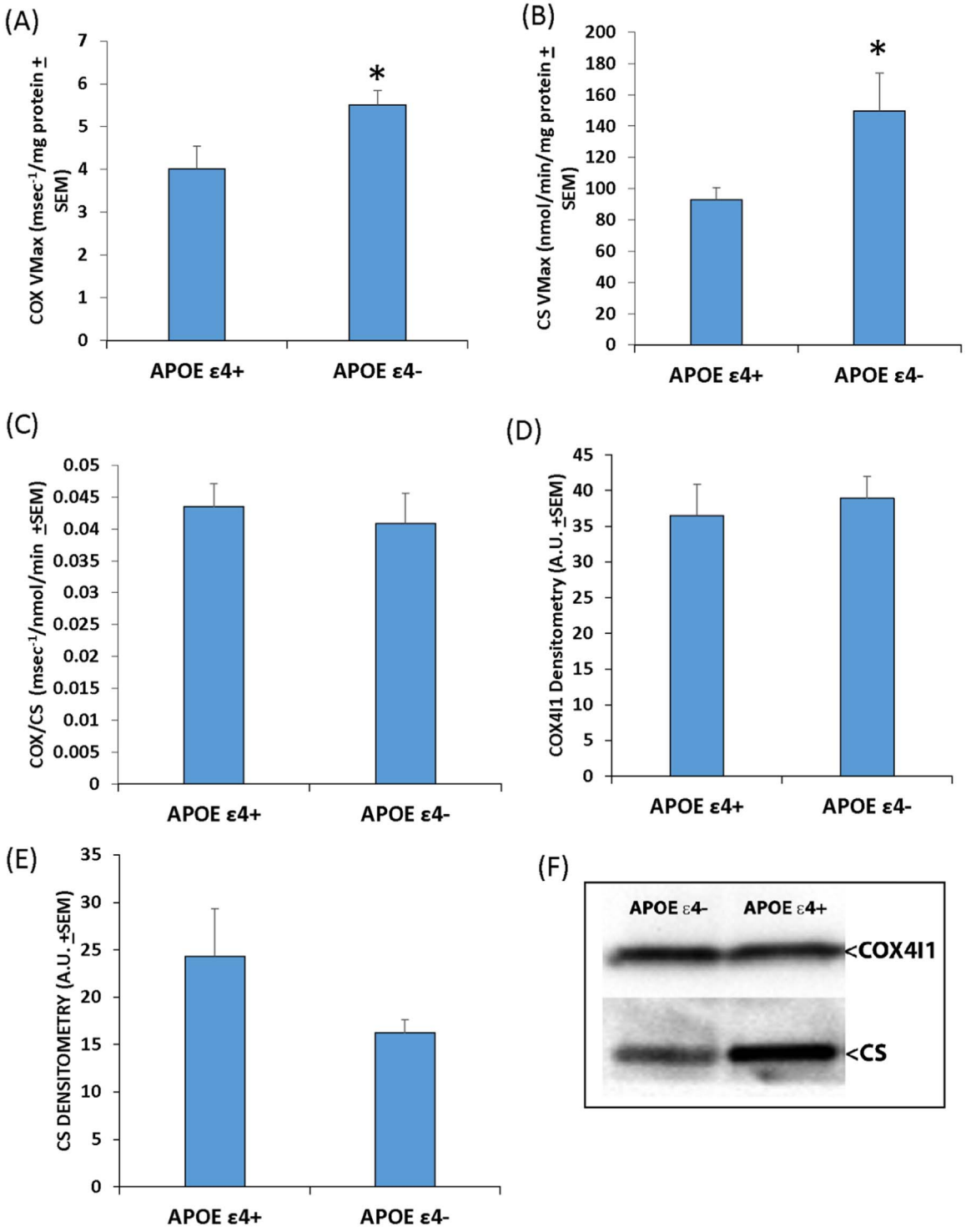

Fig. 1. Platelet mitochondria enzyme activities and protein levels in *APOE ε4* carrier and non-carrier participants. A. COX Vmax (msec^{-1}/mg). B. CS Vmax (nmol/min/mg). C. COX/CS Vmax (msec^{-1}/nmol/min). D. COX4I1 protein densitometry (A.U.). E. CS protein densitometry (A.U.). F. Representative immunoblots. For A-C, n = 8 for the *APOE ε4* carriers, and n = 7 for the non-carriers. For D and E, n = 8 for the *APOE ε4* carriers, and n = 5 for the non-carriers. *$p < 0.05$.

residual samples were available to analyze protein levels in 8 *APOE ε4* carrier and 5 *APOE ε4* non-carrier participants. The groups showed comparable COX4I1 protein levels. CS protein levels were also statistically equivalent, although we note a possible trend towards reduced CS protein in the *APOE ε4* non-carriers (Fig. 1).

Caucasians show very frequent (98%) but not exclusive *TOMM40* `*523L* and *APOE ε4* allele linkage disequilibrium, while African Americans do not [21,29]. Three of the 15 participants (20%) did not show *APOE ε4-TOMM40 L* linkage disequilibrium; two of these were African American. After regrouping our participants by *TOMM40 L* carrier (n = 7) or non-carrier (n = 8) status, we did not observe COX, CS, or COX/CS activity differences. The *TOMM40 L* groups showed comparable COX4I1 and CS protein levels, although the CS protein level trended lower in the *TOMM40 L* non-carriers (n = 6) as compared to the *TOMM40 L* carriers (n = 7) (p = 0.06) (Fig. 2).

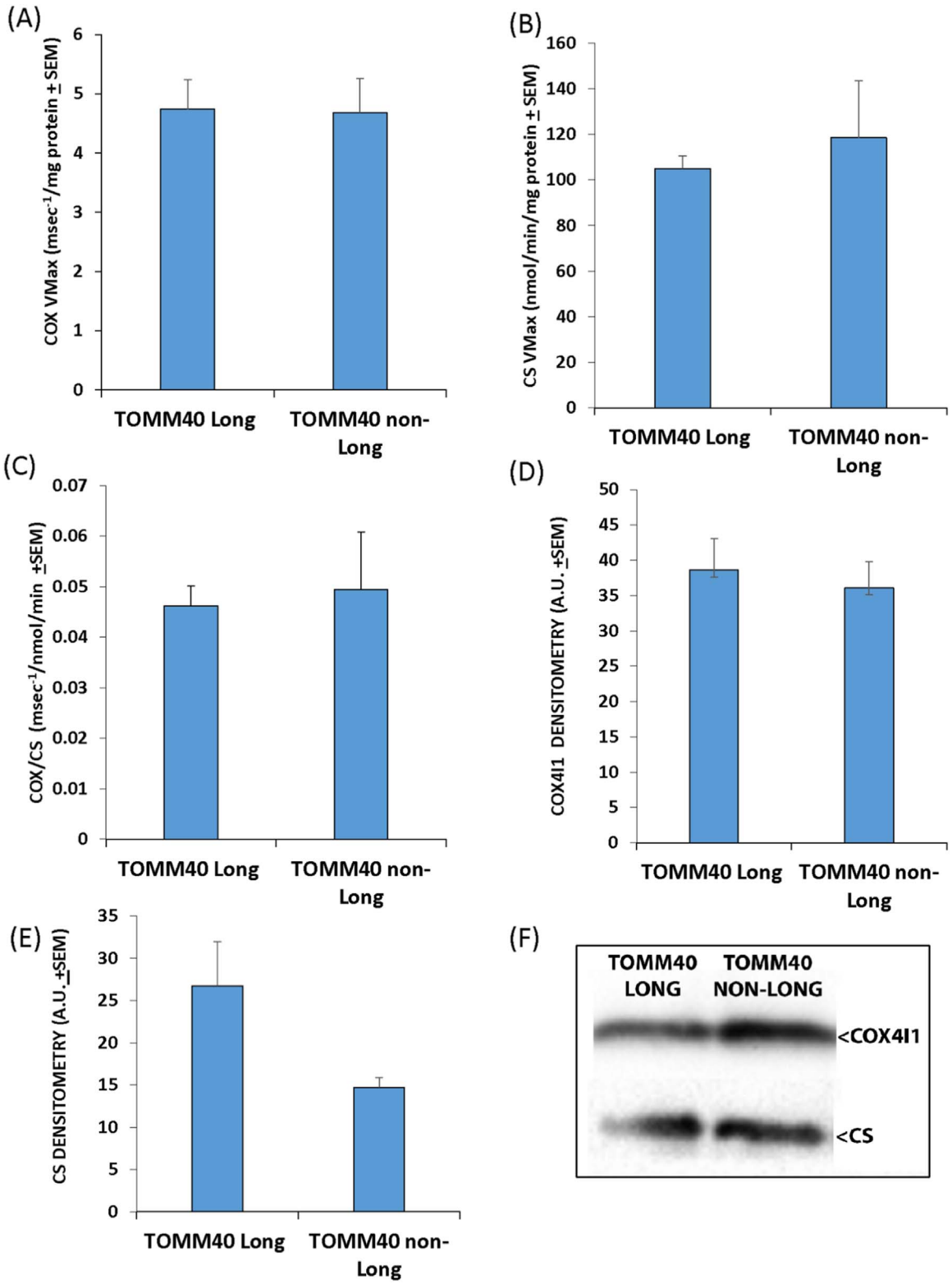

Fig. 2. Platelet mitochondria enzyme activities and protein levels in *TOMM40 L* carrier and non-carrier participants. A. COX Vmax ($msec^{-1}$/mg). B. CS Vmax (nmol/min/mg). C. COX/CS Vmax ($msec^{-1}$/nmol/min). D. COX4I1 protein densitometry (A.U.). E. CS protein densitometry (A.U.). F. Representative immunoblots. For A-C, n = 7 for the *TOMM40 L* carriers, and n = 8 for the non-carriers. For D and E, n = 7 for the *TOMM40 L* carriers, and n = 6 for the non-carriers. *$p < 0.05$.

4. Conclusions

Within a small sample of AD patients, the *APOE ε4* carriers had lower platelet mitochondria COX and CS Vmax activities than the *APOE ε4* non-carriers. Normalizing COX activities to their corresponding CS activities eliminated the COX activity difference, which likely reflects division of the lower *APOE ε4* carrier COX numerators by the lower CS denominators. COX4I1 (and by predicted extension the COX holoenzyme) and CS protein levels did not appear to mediate the lower *APOE ε4* carrier COX and CS Vmax activities. Indeed, for unclear reasons CS protein levels paradoxically trended lower in the *APOE ε4* non-carriers.

Due to our small study size these findings are preliminary and not

definitive. A consideration of several points that arise from this study could, nevertheless, assist in the planning of more definitive studies. Obviously, our data can inform power calculations. If COX activity serves as the primary outcome, normalizing the COX activity to the CS activity could potentially produce a false negative result.

Future studies should also consider potential mechanisms (or artifacts) that may produce a positive result. While our data could reflect a direct interaction of *APOE ε4*-derived apolipoprotein E protein and mitochondria, or between an *APOE ε4*-derived apolipoprotein E peptide derivative and mitochondria [8,9,22], we did not determine platelet apolipoprotein E protein levels. Even if platelets turn out not to contain apolipoprotein E protein, though, they do host apolipoprotein E receptors [30]. Signaling through these receptors reportedly affects platelet activation and aggregation states, which could indirectly influence mitochondrial function or possibly create sample preparation artifacts. Regarding the possibility that *TOMM 40L* alleles might actually mediate differences in mitochondrial function, and *APOE ε4* alleles simply indicate the presence of *TOMM 4L* alleles, our data argue against this but we must again note these remain preliminary findings and our small sample size precludes definitive conclusions.

Our sample lacked *APOE ε4* homozygous participants. This occurred by chance, not intent. We therefore do not know whether *APOE ε4* homozygosity associates with lower COX or CS activities than *APOE ε4* heterozygosity. Addressing this could provide insight into the clinical significance of the AD-*APOE*-mitochondria nexus. To this point we further note that even if *APOE* genotypes do in fact associate with lower mitochondrial enzyme activities, the extent to which this mechanistic association underlies *APOE*-determined AD risk remains unclear. Because *APOE*-related changes in mitochondrial function presumably manifest during early-life [14], and AD arises during late-life, one might argue *APOE* status primarily influences AD risk by reducing reserve enzyme capacity, thereby increasing the likelihood that age-related declines in mitochondrial function eventually surpass functional and clinically relevant thresholds.

Despite these caveats and considerations, our data suggest platelet mitochondria COX and CS activities in *APOE ε4* non-carriers may exceed those of *APOE ε4* carriers. Viewed within the context of the report of Valla et al., which found brains from young *APOE ε4* carriers showed a lower COX activity than brains from young *APOE ε4* non-carriers [14], our data are consistent with the possibility that mitochondria mediate the association between *APOE ε4* and increased AD risk [31].

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References

[1] C.C. Liu, et al., Apolipoprotein E and Alzheimer disease: risk, mechanisms and therapy, Nat. Rev. Neurol. 9 (2) (2013) 106–118.
[2] A.D. Roses, On the discovery of the genetic association of Apolipoprotein E genotypes and common late-onset Alzheimer disease, J. Alzheimers Dis. 9 (3 Suppl) (2006) 361–366.
[3] A.D. Roses, Apolipoprotein E alleles as risk factors in Alzheimer's disease, Annu Rev. Med 47 (1996) 387–400.
[4] A.D. Roses, A.M. Saunders, APOE is a major susceptibility gene for Alzheimer's disease, Curr. Opin. Biotechnol. 5 (6) (1994) 663–667.
[5] R.J. Guerreiro, D.R. Gustafson, J. Hardy, The genetic architecture of Alzheimer's disease: beyond APP, PSENs and APOE, Neurobiol. Aging 33 (3) (2012) 437–456.
[6] E.R. Martin, et al., SNPing away at complex diseases: analysis of single-nucleotide polymorphisms around APOE in Alzheimer disease, Am. J. Hum. Genet 67 (2) (2000) 383–394.
[7] J.M. Castellano, et al., Human apoE isoforms differentially regulate brain amyloid-beta peptide clearance, Sci. Transl. Med. 3 (89) (2011) 89ra57.
[8] S. Chang, et al., Lipid- and receptor-binding regions of apolipoprotein E4 fragments act in concert to cause mitochondrial dysfunction and neurotoxicity, Proc. Natl. Acad. Sci. USA 102 (51) (2005) 18694–18699.
[9] H.K. Chen, et al., Apolipoprotein E4 domain interaction mediates detrimental effects on mitochondria and is a potential therapeutic target for Alzheimer disease, J. Biol. Chem. 286 (7) (2011) 5215–5221.
[10] T. Hashimoto, et al., Apolipoprotein E, especially apolipoprotein E4, increases the oligomerization of amyloid beta peptide, J. Neurosci. 32 (43) (2012) 15181–15192.
[11] Y. Ji, et al., Apolipoprotein E isoform-specific regulation of dendritic spine morphology in apolipoprotein E transgenic mice and Alzheimer's disease patients, Neuroscience 122 (2) (2003) 305–315.
[12] J. Kim, et al., Apolipoprotein E in synaptic plasticity and Alzheimer's disease: potential cellular and molecular mechanisms, Mol. Cells 37 (11) (2014) 767–776.
[13] M. Perkins, et al., Altered Energy Metabolism Pathways in the Posterior Cingulate in Young Adult Apolipoprotein E varepsilon4 Carriers, J. Alzheimers Dis. 53 (1) (2016) 95–106.
[14] J. Valla, et al., Reduced posterior cingulate mitochondrial activity in expired young adult carriers of the APOE epsilon4 allele, the major late-onset Alzheimer's susceptibility gene, J. Alzheimers Dis. 22 (1) (2010) 307–313.
[15] E.M. Reiman, et al., Preclinical evidence of Alzheimer's disease in persons homozygous for the epsilon 4 allele for apolipoprotein E, N. Engl. J. Med. 334 (12) (1996) 752–758.
[16] G.W. Small, et al., Apolipoprotein E type 4 allele and cerebral glucose metabolism in relatives at risk for familial Alzheimer disease, JAMA 273 (12) (1995) 942–947.
[17] G. Carrieri, et al., Mitochondrial DNA haplogroups and APOE4 allele are non-independent variables in sporadic Alzheimer's disease, Hum. Genet. 108 (3) (2001) 194–198.
[18] A. Maruszak, et al., Mitochondrial haplogroup H and Alzheimer's disease–is there a connection? Neurobiol. Aging 30 (11) (2009) 1749–1755.
[19] A.D. Roses, An inherited variable poly-T repeat genotype in TOMM40 in Alzheimer disease, Arch. Neurol. 67 (5) (2010) 536–541.
[20] A.D. Roses, et al., A TOMM40 variable-length polymorphism predicts the age of late-onset Alzheimer's disease, Pharm. J. 10 (5) (2010) 375–384.
[21] A. Roses, et al., Understanding the genetics of APOE and TOMM40 and role of mitochondrial structure and function in clinical pharmacology of Alzheimer's disease, Alzheimers Dement 12 (6) (2016) 687–694.
[22] R.W. Mahley, Y. Huang, Apolipoprotein e sets the stage: response to injury triggers neuropathology, Neuron 76 (5) (2012) 871–885.
[23] R.H. Swerdlow, Mitochondria and cell bioenergetics: increasingly recognized components and a possible etiologic cause of Alzheimer's disease, Antioxid. Redox Signal. 16 (12) (2012) 1434–1455.
[24] W.D. Parker Jr, C.M. Filley, J.K. Parks, Cytochrome oxidase deficiency in Alzheimer's disease, Neurology 40 (8) (1990) 1302–1303.
[25] R.H. Swerdlow, et al., Cybrids in Alzheimer's disease: a cellular model of the disease? Neurology 49 (4) (1997) 918–925.
[26] G.M. McKhann, et al., The diagnosis of dementia due to Alzheimer's disease: recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease, Alzheimers Dement. 7 (3) (2011) 263–269.
[27] L. Mosconi, et al., Reduced mitochondria cytochrome oxidase activity in adult children of mothers with Alzheimer's disease, J. Alzheimers Dis. 27 (3) (2011) 483–490.
[28] C. Linnertz, et al., The cis-regulatory effect of an Alzheimer's disease-associated poly-T locus on expression of TOMM40 and apolipoprotein E genes, Alzheimers Dement. 10 (5) (2014) 541–551.
[29] A.D. Roses, et al., African-American TOMM40'523-APOE haplotypes are admixture of West African and Caucasian alleles, Alzheimers Dement. 10 (6) (2014) 592–601.
[30] A.M. Minihane, et al., ApoE genotype, cardiovascular risk and responsiveness to dietary fat manipulation, Proc. Nutr. Soc. 66 (2) (2007) 183–197.
[31] E.H. Corder, et al., Gene dose of apolipoprotein E type 4 allele and the risk of Alzheimer's disease in late onset families, Science 261 (5123) (1993) 921–923.