

# Blood Bioenergetic Biomarkers in Alzheimer's Disease APOE $\epsilon$ 4-Carriers

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## Research article

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# Abstract

## Background:

This study examined the impact of *APOE ε4* on blood cell bioenergetics. Prior studies have shown systemic alterations in blood cells from *APOE ε4* carriers.

## Methods:

Platelet mitochondria cytochrome oxidase (COX) and citrate synthase (CS) Vmax activities were measured in *APOE ε4* carrier and non-carrier Alzheimer's disease (AD) subjects, and lymphocyte mitochondria and bioenergetics-relevant protein and viability endpoints were measured using fresh and expanded cultures. Statistical analysis was completed using Student's T-Test.

## Results:

The mean platelet COX Vmax activity, normalized to protein content, was lower in *APOE ε4* carriers and lymphocyte Annexin V, a marker of apoptosis, was significantly higher. PINK1, a protein involved in mitophagy, was higher in *APOE ε4* carrier lymphocytes. mTOR and SIRT1, which play a role in energy sensing, were different between the groups; mTOR phosphorylation decreased while SIRT1 phosphorylation increased in *APOE ε4* carrier lymphocytes. The lipid synthesis pathway differed, as AceCSI and ATP CL increased in *APOE ε4* carrier lymphocytes, and ACC phosphorylation also increased.

## Conclusions:

These findings overall support a relationship between *APOE* genotype and bioenergetic pathways and indicate that relative to AD non-*APOE ε4* carriers, platelets and lymphocytes from AD *APOE ε4* carriers exist in a relative state of bioenergetic stress. As *APOE ε4* influences AD risk these data may help define a systemic AD biomarker phenotype.

# Background

Apolipoprotein E (*APOE*) is the strongest genetic risk factor for sporadic Alzheimer's Disease (AD). *APOE* exists as three alleles;  $\epsilon 2$  (lowers AD risk),  $\epsilon 3$  (neutral AD risk), and  $\epsilon 4$  (increases AD risk). Individuals who carry an *APOE ε4* allele are 3-4-fold more likely to develop AD, while homozygotes are 10-15-fold more likely (1). About 15-25% of the population carries an *APOE ε4* allele and 2-3% are homozygous. The exact mechanism underlying the link between AD risk and *APOE* is unknown (1).

The *APOE* gene product, apolipoprotein E (APOE), is a lipid binding protein (i.e. lipoprotein) which functions in cholesterol metabolism. Within the brain, APOE is the main cholesterol carrying protein where it is mostly expressed by astrocytes and transports cholesterol to neurons (1). Allele variants lead to one or two amino acid substitutions in the protein product (APOE  $\epsilon 2$ , cys112, cys158; APOE  $\epsilon 3$ , cys112, arg158; and APOE  $\epsilon 4$ , arg112, arg158). The presence of arg112 in APOE  $\epsilon 4$  causes an alternative folding

of the peptide that gives rise to a cleavage site from which a mitochondrial-toxic C-terminal fragment is formed (1-3). APOE also functions to modulate inflammation and amyloid beta clearance (4-6).

*APOE* genotype is associated with early changes (i.e. prior to dementia onset) in brain metabolism, cognition, and neuroimaging measures (7-14). Systemic metabolic affects are also observed (15). We previously reported changes in blood based mitochondrial biomarkers in women *APOE*  $\epsilon 4$  carriers with AD. When compared to age matched non-carrier women with AD, the AD *APOE*  $\epsilon 4$  carriers had decreased platelet cytochrome oxidase (COX) and citrate synthase (CS) enzyme maximum velocities ( $V_{max}$ ) (16).

A goal of this study was to expand and extend our prior study to include male AD subjects (16). We also sought to further our findings by measuring a range of metabolic outcomes in blood cells and compared some endpoints to post-mortem brain samples. These blood-based mitochondrial biomarkers were used as primary and target engagement outcomes in recent clinical trials (17-19). This report summarizes findings from human platelets and lymphocytes. The platelet analysis was conducted as part of the Sequel in AD 2 (SEAD2) clinical trial (ClinicalTrials.gov Identifier: NCT03101085), and the lymphocyte analysis is part of the "White Blood Cell Endpoints in AD" (WEAD) study we designed as an add-on to the SEAD2 study.

## Materials And Methods

### *Approvals and Human Subjects*

The Kansas University Medical Center Human Subjects Committee (KUMC HSC) approved all human subject participation 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 participants who met McKhann et al. AD diagnostic criteria (20). Participants were excluded 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. At the beginning of the study the trial participants underwent a 40 ml phlebotomy.

Autopsy brain samples were obtained from the KU Alzheimer's Disease Research Center (KU ADRC) Neuropathology Core. The KU ADRC maintains a clinical cohort and collects brains from consenting cohort decedents. The autopsy consent process is approved by the KU HSC.

### *Phlebotomy and Blood Cell Separation*

Forty ml of blood was collected in tubes containing acid-citrate-dextrose anticoagulant. One ml of whole blood was removed and stored at  $-80^{\circ}\text{C}$  for genotyping; the rest was used for platelet and lymphocyte harvesting.

Fifteen ml of Histopaque 1077 was centrifuged in an AccuSpin tube for 1 minute at  $1700 \times g$ . Blood was layered on top of the AccuSpin tube frit and centrifuged for 15 minutes at  $400 \times g$ . Platelet-rich plasma and the buffy coat were collected in separate tubes and pelleted by centrifugation for 15 minutes at  $1700$

x g. The pellets were washed with phosphate buffered saline (PBS) and re-centrifuged. Platelets were used for mitochondrial isolation (see below). Lymphocytes were used for flow cytometry biomarkers and tissue culture expansion (see below). Blood samples were processed on the same day as the blood draw.

### *Mitochondrial Isolation*

Platelets were 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 minutes. The ruptured platelets were centrifuged at 1000 x g for 15 minutes, 4°C. The supernatant was transferred to a new tube, while the pellet (intact platelets) was resuspended in MSHE buffer and subjected to nitrogen cavitation for a second time (1200 psi for 20 minutes). Both supernatants were combined and centrifuged at 12,000 x g for 10 minutes, 4°C. The resulting mitochondrial pellet was resuspended in MSHE buffer.

### *COX and CS Vmax Assays*

We added aliquots of the enriched platelet 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 ( $\text{msec}^{-1}$ ). For the CS Vmax, we followed the formation of 5-thio-2-nitrobenzoate (nmol/min) (21). The COX rate was normalized to mg protein ( $\text{msec}^{-1}/\text{mg protein}$ ) or to the CS rate (yielding a value with units of  $\text{msec}^{-1}/\text{nmol/min}$ ), 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.

### *Dye-Based Assays and Flow Cytometry*

Fresh lymphocytes were suspended in HBSS (with  $\text{Ca}^{2+}/\text{Mg}^{2+}$ ) at  $1 \times 10^6/\text{mL}$ . Four mL of lymphocytes were used for negative (no stain), JC1, MitoSox, and Mitotracker/Annexin V staining. For staining we used 10  $\mu\text{L}$  of 200  $\mu\text{M}$  JC1, 4  $\mu\text{L}$  of 10  $\mu\text{M}$  Mitotracker Red, 2  $\mu\text{L}$  of 5 mM MitoSox, or no dye. Cells were incubated at 37°C with 5%  $\text{CO}_2$  for 30 minutes. All samples were centrifuged to pellet cells (1700 x g for 5 minutes). Cells were washed with HBSS and centrifuged again (1700 x g for 5 minutes). The supernatants were removed and 500  $\mu\text{L}$  of HBSS was added to each tube (for all samples except the MitoTracker/Annexin V samples). For MitoTracker/Annexin V samples, 100  $\mu\text{L}$  of 1X Annexin V binding buffer was added with 5  $\mu\text{L}$  of Annexin V dye. MitoTracker/Annexin V samples were incubated at room temperature for 15 minutes, following which 400  $\mu\text{L}$  of 1X Annexin V binding buffer was added. All samples were placed on ice and immediately analyzed using an LSRII flow cytometer (BD Bioscience).

### *Lymphocyte Culture*

Lymphocytes were resuspended in complete RPMI medium (RPMI, 10% FBS, Pen/Strep, 20 U/mL IL-2, and 20 ng/mL CD3) at  $1 \times 10^6$  cells/mL in a T75 culture flask. Cells were fed every other day and split as needed to keep cell concentrations at  $1 \times 10^6$  cells/mL. Cells were used for immunocytochemistry as described below after seven days of culture.

## *Immunochemistry*

For expanded lymphocyte cultures and autopsied human brain superior frontal gyrus tissue sections, protein was collected in RIPA buffer with protease/phosphatase inhibitors (ThermoFisher). Equal protein amounts were resolved by SDS-PAGE (Criterion TGX gels, BioRad) and proteins were transferred to PVDF membranes (ThermoFisher). Immunoblots were completed and antibodies are listed in Table 1. To visualize bands, we used WestFemto Super Signal HRP Substrate (ThermoFisher) and a ChemiDoc XRS imaging platform.

## *APOE Genotyping*

We used a single nucleotide polymorphism (SNP) allelic discrimination assay to determine *APOE* genotypes. This involved adding 5 ul 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*  $\epsilon 2$ ,  $\epsilon 3$ , and  $\epsilon 4$  alleles.

## *Data and Statistical Analyses*

We organized the data from the participants into two groups, one in which participants had at least one *APOE*  $\epsilon 4$  allele, and one in which they did not. We compared means by two-way Student's T-tests with significance defined as  $p < 0.05$ .

## **Results**

Table 2 summarizes subject demographics. As we found in our prior study, *APOE*  $\epsilon 4$  carriers have lower platelet mitochondrial COX Vmax activities when compared to non-carriers ( $\epsilon 3/\epsilon 3$ ) (Figure 1). Unlike our prior study, we found no change in CS Vmax with *APOE* genotype. Supplemental data table 1 (Table S1) summarizes enzyme Vmax findings by individual genotypes (heterozygotes versus homozygotes).

Lymphocytes from *APOE*  $\epsilon 4$  carriers and non-carriers showed similar levels of mitochondrial superoxide production (MitoSox), mitochondrial membrane potential (JC1), and mitochondrial number (MitoTracker). These data are summarized in Table S2 (supplemental data). We observed an increase in apoptotic lymphocytes from *APOE*  $\epsilon 4$  carriers versus non-carriers ( $\epsilon 3/\epsilon 3$ ) (Figure 2). Supplemental data table 3 (Table S3) examines the platelet mitochondria and fresh lymphocyte biomarkers by sex in these AD subjects. In addition to women, we can now report biochemical phenotypes extend to men with AD. Comparing women to men revealed only one difference, which was women had a significantly higher lymphocyte mitochondrial membrane potential.

To facilitate lymphocyte protein expression assays we expanded them in culture. Levels of PINK1, a protein involved in mitophagy, was higher in *APOE*  $\epsilon 4$  carrier lymphocytes. mTOR and SIRT1, which play a role in energy sensing, were different between groups. Specifically, mTOR phosphorylation decreased while SIRT1 phosphorylation increased in *APOE*  $\epsilon 4$  carrier lymphocytes. We also observed changes in the

lipid synthesis pathway. AceCSI and ATP CL increased, and ACC phosphorylation increased (Figure 3-4). For data summarizing all proteins examined see supplemental data table 4 (Table S4).

The change in ACC phosphorylation appeared especially robust, which prompted us to examine this parameter in the superior frontal gyrus of post-mortem AD and control human brains. *APOE ε4* carrier post-mortem human brain samples showed increased pACC regardless of whether the subject donor carried an AD diagnosis (Figure 4).

## Discussion

Here we reaffirm a smaller study (16) that found lower platelet COX activity in women with AD and an *APOE ε4* allele, versus those without an *APOE ε4* allele. We further extend this finding to men with AD and an *APOE ε4* allele. We did not replicate the smaller study's finding of lower platelet CS Vmax activities in the *APOE ε4* carriers.

COX Vmax is lower in AD subjects, a finding replicated in brain, fibroblasts, and blood cells (14, 16, 21-29). Mechanistic studies show that COX is assembled differently in AD cohorts, which may contribute to Vmax deficits (30). Additional studies associate the COX deficit with changes to its mRNA expression within brain (31, 32). It is apparent that the COX deficit is attributed to at least some extent to mitochondrial DNA, either through inheritance or somatic mutations (21, 33). Deficits in COX functionality will lead to bioenergetic stress including changes to redox balance and ATP production (24, 33). As a systemic biomarker, reduced platelet COX Vmax correlates strongly with brain glucose metabolism (34). Future studies should leverage this systemic biomarker to understand the origins of bioenergetic stress observed in AD.

To extend our biomarker observations, we utilized lymphocytes from the same blood draw. *APOE ε4* carriers showed an increase in pSIRT1, PINK1, AceCS1, ATP CL, and pACC levels; a decrease in pmTOR; and an increase in Annexin V staining. We found no changes in lymphocyte mitochondrial mass, mitochondrial membrane potential, or mitochondrial superoxide using our methodologies. We did see a sex difference in the lymphocyte mitochondrial membrane potential, in which female AD subjects had higher mitochondrial membrane potentials compared to male AD subjects.

Lymphocyte apoptosis can be attributed to "neglect" or loss of extrinsic signals, a process that occurs through mitochondrial energy failure and the loss of anapleurosis (35-39). Bioenergetic stress, as a consequence of reduced glucose metabolism, may play a role in lymphocyte apoptosis (37, 38, 40). Our overall findings suggest increased lymphocyte apoptosis may reflect a consequence of bioenergetic stress.

A previous study claimed SIRT1 phosphorylation at the site we interrogated reflects SIRT1 activation (41). SIRT1 regulates chromatin remodeling, allowing for gene expression changes that adapt to stress (42). SIRT1 functions to alter cell metabolism including glycolysis flux, lipid homeostasis, insulin secretion,

and inflammation. Energy stress activates SIRT1, which essentially serves as a stress response master regulator (42-46).

mTOR promotes cell growth. It activates under anabolic conditions that coincide with energy-sufficient states and deactivates under catabolic conditions of energy stress. Serine 2448 phosphorylation levels positively correlate with mTOR activity, and suggest a downstream stimulation of mTORC2 and mTORC1, protein complexes implicated in cell metabolic regulation (47, 48). Decreased mTOR 2448 phosphorylation in *APOE ε4* carrier lymphocytes suggests that allele shifts the anabolic-catabolic balance to a more catabolic setting.

Cells experiencing catabolic shifts typically increase autophagy, a process of internal digestion that replenishes raw molecular materials. PINK1 helps mediate autophagy and increased PINK1 suggests increased mitophagy/autophagy flux (49). Furthermore, mTOR modulates autophagy through ULK1 (unc-51 like kinase 1 or ATG1), as inhibition of mTOR during nutrient starvation leads to activation of ULK1 and autophagy (50). In this case, elevated PINK1 and reduced mTOR activation in AD *APOE ε4* carrier lymphocytes suggests some degree of cell-level energy stress in the *APOE ε4* carriers.

The most robust finding in lymphocytes was the increase in pACC and ACC expression by *APOE* genotype. ACC is an enzyme which converts acetyl coA into malonyl coA through carboxylation, which represents an early integral step in fatty acid synthesis. ACC phosphorylation inhibits its activity and turns off lipid biosynthesis. To understand if this change was specific, we also examined ACC expression and phosphorylation in human post-mortem brain samples. We found that pACC levels also increased in brains from AD and *APOE ε4* carriers.

AceCS1 is a cytosolic enzyme that catalyzes the conversion of acetate and CoA to acetyl-CoA, where it enters lipid synthesis. SIRT1 reportedly regulates its activity (46). ATP CL converts citrate to acetyl CoA and oxaloacetate, and links carbohydrate and fatty acid metabolism. Both ATP CL and AceCS1 expression are higher in *APOE ε4* carriers, which could represent a cause or consequence of the observed ACC changes. Increased AceCS1 and ATP CL could potentially increase if an ACC-mediated reduction in lipid biosynthesis leads to a secondary increase in acetyl coA. Based on this pattern of observations, investigating acetyl CoA and its up/downstream metabolites in *APOE ε4* carriers could prove informative.

Our data indicate that compared to AD *APOE ε4* non-carriers, AD *APOE ε4* carrier lymphocytes exhibit a relative state of bioenergetic stress and catabolic shift. We already know from fluorodeoxyglucose positron emission tomography (FDG PET) studies that brains from cognitively normal, middle-aged *APOE ε4* carriers show reduced glucose utilization (11, 13, 51, 52), but clearly this *APOE*-dependent metabolic phenotype extends beyond the brain.

The *APOE ε4* allele also associates with an increased burden of brain white matter hyperintensity (WMH) (7, 53-56). Speculated causes of WMHs include microvascular disease, defective myelin, gliosis, inflammation, neurodegeneration, or a combination of these factors. Neuroimaging studies suggest *APOE ε4* carriers have increased myelin breakdown and these effects can be found in infants during

brain development (57-59). Myelin represents a sizeable brain lipid depository, and in a state of bioenergetic stress or starvation the brain may switch towards a more catabolic state that features myelin consumption over synthesis (60).

The literature emphasizes hepatocytes, astrocytes, and macrophages, but not lymphocytes and platelets, express the *APOE* gene. The presence of an *APOE*-associated molecular phenotype in lymphocytes, which others also report (61), and platelets warrants consideration (16). Perhaps circulating APOE protein influences these cells. Under stress conditions neurons will increase *APOE* expression, which raises the question of whether other stressed cell types demonstrate this behavior. Maybe even low levels of expression can impact a cell. Myeloid APOE can alter lymphocyte biology through non-cell autonomous signaling events (62). Other genes with variants in linkage disequilibrium with the *APOE* isoforms, such as *TOMM40*, could also play a role (63).

## Conclusion

Despite years of research, the mechanisms that underlie the AD-*APOE* association remain unclear. Our data support the view that bioenergetic metabolism-related stresses may mediate this. As APOE expression occurs outside the brain and is functionally important outside the brain, it seems reasonable to propose a systemic AD phenotype may exist. This could manifest as subtle metabolic shifts of the type we now demonstrate and could perhaps explain other associations including reported connections between dementia and type II diabetes mellitus (64). This study also argues peripheral metabolism biomarkers may reflect brain metabolism. If further validated, peripheral biomarkers like the ones we now show, or those developed by others (65), could provide valuable insight into AD biology, suggest treatment strategies, and monitor target engagement in clinical trials.

## Abbreviations

AD, Alzheimer's Disease; APOE, Apolipoprotein E; COX, cytochrome oxidase; CS, citrate synthase; DNA, deoxyribonucleic acid; FDG-PET, fluorodeoxyglucose positron emission tomography; HBSS, Hank's balanced salt solution; HSC, human subjects committee (or IRB, institutional review board); KU ADRC, University of Kansas Alzheimer's Disease Research Center; KUMC, University of Kansas Medical Center; MSHE, Mannitol/sucrose/HEPES/EDTA buffer; mTORC1/2, mammalian target of rapamycin complex 1/2; RIPA, Radioimmunoprecipitation assay buffer; RPMI, Roswell Park Memorial Institute Medium; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SEAD2, S-equol in AD 2 (clinical trial name); SNP, single nucleotide polymorphism; TOMM40, translocase of the outer mitochondrial membrane 40; ULK1, unc-51 like kinase 1 or ATG1; Vmax, maximum velocity; WEAD, White-Blood Cell End-points in AD (study name); WMH, white matter hyperintensity All other protein names are defined in table 1.

## Declarations

**Ethics approval and consent to participate.** The Kansas University Medical Center Human Subjects Committee (KUMC HSC) approved all human subject participation 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).

**Consent for publication.** NA

**Availability of data and materials.** The datasets generated and/or analysed during the current study are not publicly available due to restrictions with our HSC approval but are available from the corresponding author on reasonable request.

**Competing interests.** The authors declare that they have no competing interests.

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**Authors' contributions.** HMW obtained funding for the work, completed the work, and wrote the manuscript. SJM, SJK, XW, and BWM assisted with data collection. RB, AMB, HA, and ES consented and recruited participants. JMB and RHS assisted with HSC approval. RHS edited manuscript.

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## Tables

<b>Table 1. Antibodies. p=phosphorylated.</b>		
<b>Protein Target</b>	<b>Protein Acronym</b>	<b>Company/Product Number</b>
Acetyl-CoA carboxylase	ACC/pACC	Cell Signaling 3676/11818
acetyl-CoA synthase 1	AceCS1	Cell Signaling 3658
long-chain acyl-CoA synthetase	ACSL1	Cell Signaling 9189
Beta Actin	Actin ( $\beta$ )	Cell Signaling 3700
ATP citrate lyase	ATP CL/pATP CL	Cell Signaling 4332/4331
BCL2 Interacting Protein 3 Like	Bnip3L	Cell Signaling 44060
Cleaved Caspase 3	-	Cell Signaling 9664
Cytochrome oxidase subunit 4 isoform 1	Cox411	ThermoFisherMA5-15078
Citrate Synthase	CS	Cell Signaling 14309
Fatty Acid Synthase	-	Cell Signaling 3180
Heat Shock Protein 27	pHSP27	Cell Signaling 9709
c-Jun N-terminal kinase	pJNK	Cell Signaling /4668
Lipin	-	Cell Signaling 14906
Cytochrome c oxidase subunit 2	mtCO2	Abcam 79393
mammalian target of rapamycin	mTOR/pmTOR	Cell Signaling 2983/5536
NADH dehydrogenase 1	ND1	Abcam 181848
NuclearFactor kappa-light-chain-enhancer of activated B cells	NfKB	Cell Signaling 8242
Parkin	-	Cell Signaling 4211
PTEN-induced kinase 1	PINK1	Cell Signaling 6946
Sirtuin 1	pSIRT1/SIRT1	Cell Signaling 2314/2493
Superoxide Dismutase 2	SOD2	Cell Signaling 13141
Mitochondrial transcription factor A	TFAM	Abcam 131607

<b>Table 2. Demographics</b>		
<b>Systemic Biomarker Samples</b>		
	<b>Sex (M/F)</b>	<b>Age</b>
<i>APOE</i> ε4 carriers	17/15	73.9 (8.5)
<i>APOE</i> Non-carriers	12/10	74.6 (7.4)
<i>APOE</i> ε4/ε4	4/5	71.6 (8.6)
<i>APOE</i> ε3/ε4	13/9	74.2 (8.2)
<i>APOE</i> ε3/ε3	10/9	74.5 (7.1)
<i>APOE</i> ε2 heterozygous	2/2	77.5 (11.6)
<b>Human Autopsy Brain Samples</b>		
	<b>Sex (M/F)</b>	<b>Age</b>
ND	5/2	79.3 (9.3)
AD	5/3	78.9 (9.2)
<i>APOE</i> ε4 carriers	4/3	81.3 (7.7)
<i>APOE</i> Non-carriers	6/2	77.1 (9.8)
<b>Mean (standard deviation)</b>		

## Figures

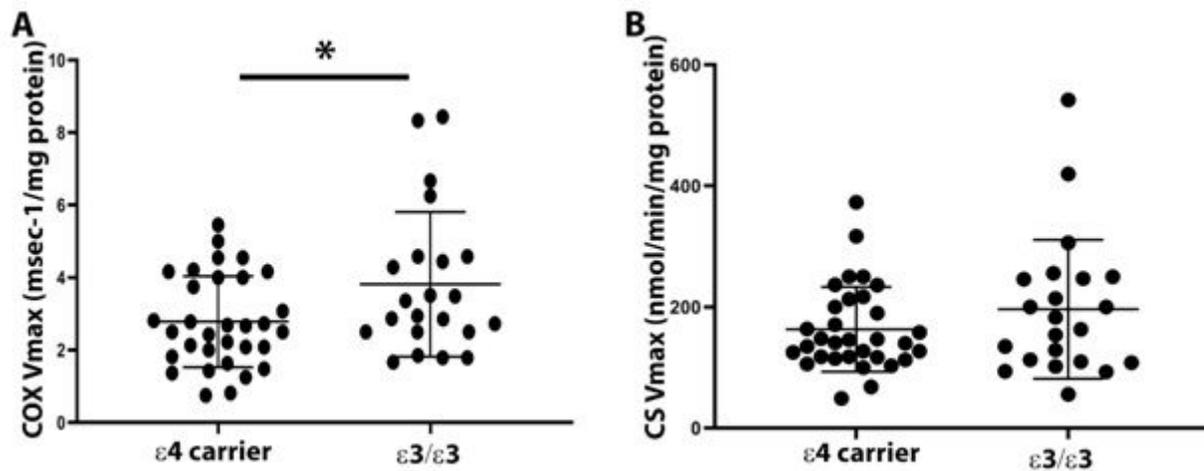


Figure 1

Platelet mitochondria enzyme Vmax Biomarkers. Platelet mitochondria were isolated and enzyme Vmax activities measured as described in materials and methods. A. COX Vmax by APOE genotype ( $\epsilon 4$  carriers versus  $\epsilon 3/\epsilon 3$ ) B. CS Vmax by APOE genotype ( $\epsilon 4$  carriers versus  $\epsilon 3/\epsilon 3$ ) \* indicates  $p < 0.05$ , \*\* indicates  $p < 0.01$ . Data are shown as mean  $\pm$  SEM.

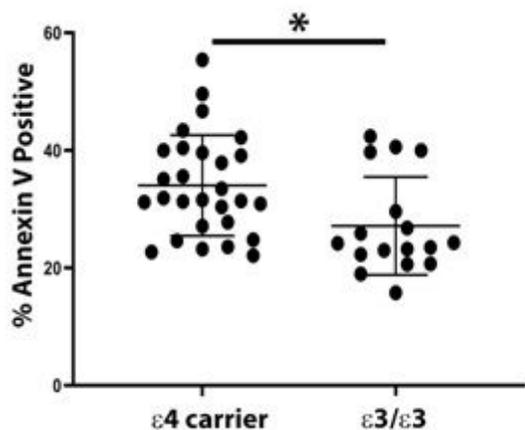
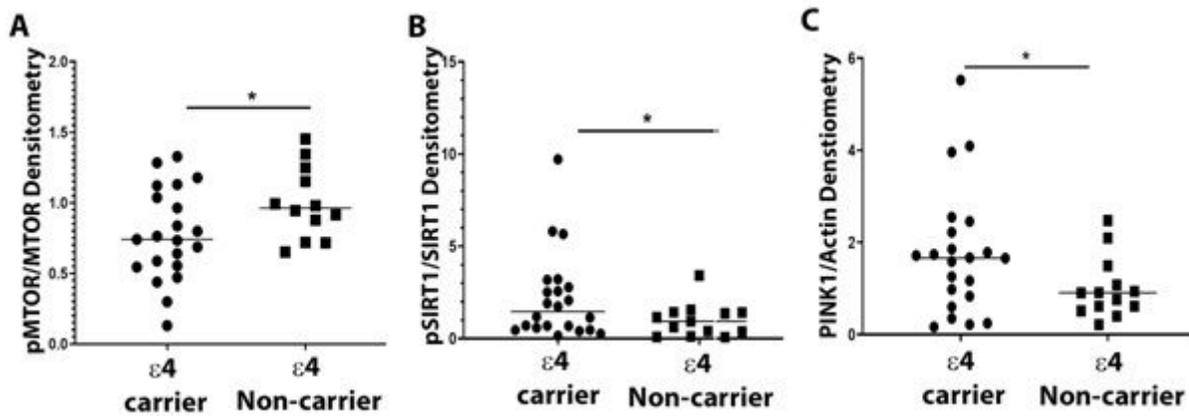


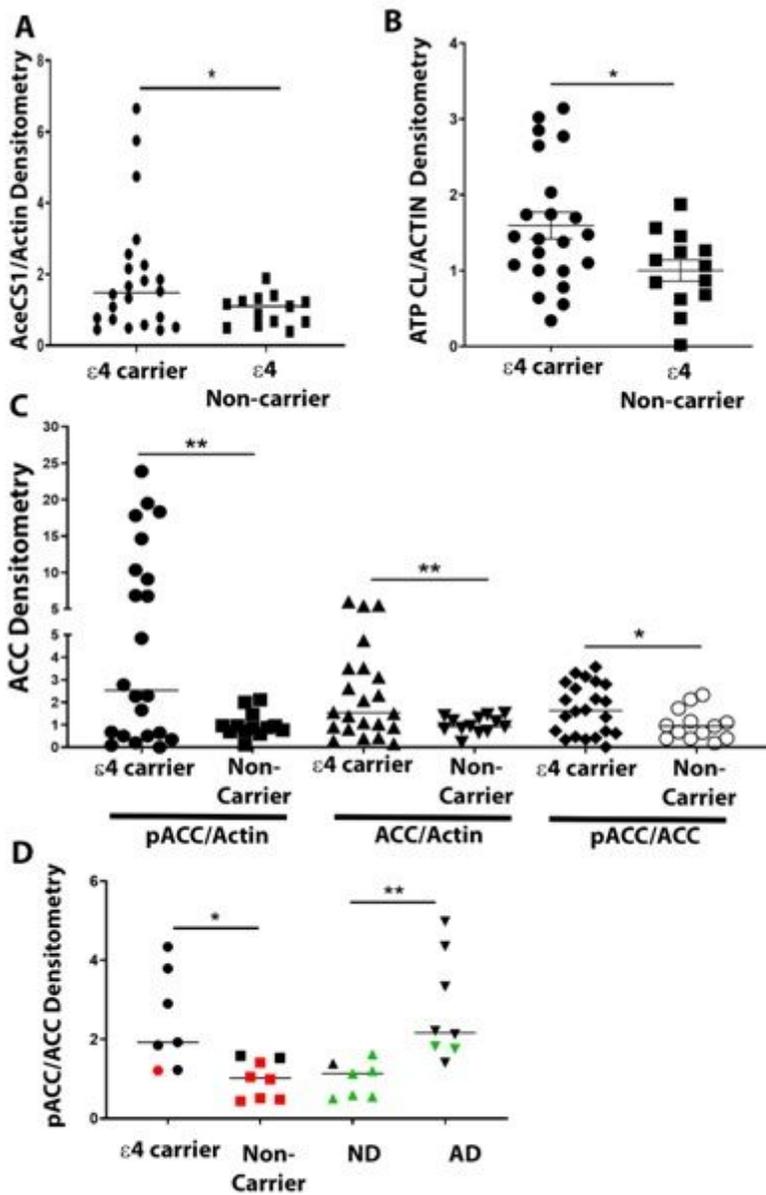
Figure 2

Fresh Lymphocyte Apoptotic Biomarker. Lymphocytes were isolated and stained for Annexin V as described in materials and methods. % lymphocytes positive for Annexin V by APOE genotype ( $\epsilon 4$  carriers versus  $\epsilon 3/\epsilon 3$ ). \* indicates  $p < 0.05$ .



**Figure 3**

Cultured Lymphocyte Energy Sensing pathway protein expression. Lymphocytes were lysed and assayed for protein expression as described in materials and methods. A. Lymphocyte pMTOR/MTOR densitometry by APOE genotype. B. Lymphocyte pSIRT1/SIRT1 densitometry by APOE genotype. C. Lymphocyte PINK1/ACTIN densitometry by APOE genotype \* indicates  $p < 0.05$ . Data are shown as mean  $\pm$  SEM.



**Figure 4**

Cultured Lymphocyte and Autopsy Human Brain lipid signaling pathway protein expression. Lymphocytes (or autopsied human brain) were lysed and assayed for protein expression as described in materials and methods. A. Lymphocyte AceCS1/ACTIN densitometry by APOE genotype B. Lymphocyte ATP CL/ACTIN densitometry by APOE genotype C. Lymphocyte ACC densitometry by APOE genotype E. ACC densitometry in autopsied human brain samples by APOE genotype or diagnosis. Red indicates ND subjects when data are separated by APOE genotype. Green indicates APOE  $\epsilon 4$  Non-Carriers when data are separated by diagnosis. \* indicates  $p < 0.05$ , \*\* indicates  $p < 0.01$ . Data are shown as mean  $\pm$  SEM.

## Supplementary Files

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