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Altered Energy Metabolism Pathways in the Posterior Cingulate in Young Adult Apolipoprotein E ε4 Carriers

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Abstract. The *APOE* gene, encoding apolipoprotein E, is the primary genetic risk factor for late-onset Alzheimer's disease (AD). Apolipoprotein E ε4 allele (*APOE4)* carriers have alterations in brain structure and function (as measured by brain imaging) even as young adults. Examination of this population is valuable in further identifying details of these functional changes and their association with vulnerability to AD decades later. Previous work demonstrates functional declines in mitochondrial activity in the posterior cingulate cortex, a key region in the default mode network, which appears to be strongly associated with functional changes relevant to AD risk. Here, we demonstrate alterations in the pathways underlying glucose, ketone, and mitochondrial energy metabolism. Young adult *APOE4* carriers displayed upregulation of specific glucose (GLUT1 & GLUT3) and monocarboxylate (MCT2) transporters, the glucose metabolism enzyme hexokinase, the SCOT & AACS enzymes involved in ketone metabolism, and complexes I, II, and IV of the mitochondrial electron transport chain. The monocarboxylate transporter (MCT4) was found to be downregulated in *APOE4* carriers. These data suggest that widespread dysregulation of energy metabolism in this at-risk population, even decades before possible disease onset. Therefore, these findings support the idea that alterations in brain energy metabolism may contribute significantly to the risk that *APOE4* confers for AD.

Keywords: Alzheimer's disease, APOE, biomarker, energy metabolism, glucose, ketones, mitochondria, monocarboxylate, neurodegeneration, posterior cingulate

INTRODUCTION

Alzheimer's disease (AD) is the most common cause of dementia and the sixth-leading cause of death in the United States [1]. The prevalence of this devastating and costly disease is expected to more than triple worldwide by 2040 [2], highlighting the need to develop an improved understanding of its pathophysiology. *APOE* is the primary genetic factor associated with late-onset AD, which comprises the vast majority of AD cases [3]. There are three major *APOE* alleles in humans: ε2 (frequency ∼6%; associated with decreased risk for AD), ε3 (∼78%; neutral

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risk), and ε4 (∼15%; increased risk) [4–6]. The effect of the apolipoprotein ε4 allele (*APOE4)* on disease status is dramatic, with *APOE4* homozygotes having up to 15 times, and *APOE4* heterozygotes up to 4 times, the risk for AD when compared to risk-neutral *APOE3* homozygotes [3, 7]. Despite this influence, the role of *APOE*, which encodes apolipoprotein E (apoE), in AD remains incompletely understood, although there is much evidence that it is pleiotropic and may exert an influence in AD pathophysiology via effects on metabolism, synaptic function, neurodevelopment, inflammation, and amyloid-β (Aβ) aggregation and clearance [8–12]. Due to this clear impact of *APOE* genotype on AD risk, investigation of young adult *APOE4* carriers (who are decades from possible disease onset) represents a valuable opportunity to examine physiological alterations that may increase vulnerability to AD and therefore provide unique insight into the pathophysiology of the disease.

Brain imaging research has previously described significant functional and structural variations in *APOE4* carriers across the lifespan, although results in certain imaging modalities (e.g., functional MRI) have not always been consistent, possibly due to methodological differences [13–16]. The use of FDG PET imaging, which measures the cerebral metabolic rate for glucose (CMRglu) has consistently demonstrated a pattern of decrements in CMRglu (hypometabolism) in AD patients [14]. Notably, defects in energy metabolism are known to influence AD pathophysiology [17, 18]. Additional work in *APOE4* carriers has identified similar patterns of hypometabolism in the late-middle aged, who also show longitudinal declines in CMRglu, and in young adults [19–21], making CMRglu a strong marker for AD-related physiological vulnerability. Similarly, others at risk for AD also display altered patterns of metabolism [22]. The posterior cingulate cortex (PCC), a functional hub in the default mode network (DMN), a brain network highly active at rest, exhibits consistent metabolic decrements in *APOE4* carriers. Imaging studies demonstrate that the PCC has high energy demand and is among the brain regions that make heavy use of glycolysis, particularly aerobic glycolysis, which is defined as glucose utilization in excess of that used for oxidative phosphorylation despite sufficient available oxygen [23, 24]. Interestingly, there is substantial overlap between regions of the DMN, utilization of aerobic glycolysis, and amyloid deposition, further pointing to the PCC as a candidate region for detailed investigation of metabolic dysfunction leading to AD susceptibility [25–27]. In an attempt to resolve early brain alterations associated with AD susceptibility in the PCC, we previously examined PCC tissue from young adult *APOE4* carriers and found reductions in mitochondrial oxidative function in the superficial cortical layers in the absence of apparent Aβ or tau pathology [28]. These superficial layers contain many dendritic tufts of deeper neurons (e.g., layer III pyramidal), and thus these reductions in mitochondrial oxidative function might relate to synaptic or local metabolic alterations. Notably, apoE4 has been linked to metabolic dysfunction [11, 29] and also to altered synaptic development and function [11, 30] in cell culture and animal studies. Importantly, these PCC histochemistry results in young adult *APOE4* carriers mirrored our earlier study of AD patients, who demonstrated superficial laminar metabolic deficits across the neocortex and most strongly in the PCC [31, 32], again hinting at alterations in metabolic function playing a role in AD risk.

As the human brain utilizes up to 20% of the total energy used by the body, multiple methods of energy metabolism are employed to maintain its function [33–36]. Depending on context, the brain may variably utilize pathways involved in aspects of glucose metabolism, ketone metabolism, and mitochondrial oxidative function variably. Therefore, a central and outstanding question is how specific energy metabolism pathways, which are tightly coupled to synaptic function and neural efficiency, may be altered in *APOE*4 carriers. These alterations represent functional changes linked to *APOE4* status, and further, they may function as a physiological substrate for AD risk. Here, we survey the metabolic pathways underlying glucose, ketone, and mitochondrial energy metabolism in the AD-vulnerable PCC in order to examine the differential effects of *APOE4* status on these physiological parameters in a novel population of young adult subjects.

MATERIALS AND METHODS

Frozen blocks of PCC tissue were obtained from the University of Maryland Brain and Tissue Bank, which is a brain and tissue repository of the NIH NeuroBioBank. The samples analyzed represent a subset of samples from our previous work [28], matched to minimize group differences in postmortem interval and age between the *APOE4* carriers and non-carriers.

For relative quantitation of select protein targets, small samples were cut from each frozen cortical tissue block, quickly weighed, and placed in a chilled Dounce glass-glass homogenizer with cold RIPA buffer (150 mM NaCl, 1.0% NP-40, 1.0% SDS, 50 mM Tris HCl, and HALT protease inhibitor cocktail [Pierce]) and homogenized using a minimum of 20 pestle strokes. Samples were rotated at 4°C for 30 min, and then centrifuged at 14100 RCF for 15 min at 4°C. The clarified supernatant was aliquoted to multiple chilled tubes and stored at –80°C until analyzed. Protein quantification in each sample was via detergent-compatible (DC) Lowry assay (BioRad) per manufacturer's instructions, and 25 μg protein/lane was loaded into 4–12% Bis-Tris mini-gels (Life Technologies). Gels were run at 125–150V for approximately 2 h under denaturing and reducing conditions, then proteins were transferred to nitrocellulose membrane. Transfer efficiency was verified via Ponceau S staining and membranes were blocked with 5% non-fat dry milk or BSA in Tris-buffered saline (50 mM Tris-base, 0.9% NaCl) with 0.01% Tween 20 (Fisher; TBST). Primary antibodies were suspended in 5% non-fat dry milk in TBST; HRP-conjugated and DyLight-conjugated secondary antibodies were suspended in 5% non-fat dry milk or BSA in TBST with 0.01% SDS. Blots labeled with HRP-conjugated antibodies were developed with chemiluminescent reagent (Pierce) and imaged on film. Images of the film were captured with a high-resolution CCD camera mounted over a fiber-optic backlight under uniform lighting conditions. Blots labeled with DyLight-conjugated antibodies were imaged on an Odyssey Infrared Imaging System (LI-COR). Bands were checked for saturation using a thresholding function and analyzed in Image Studio (Li-Cor) software.

Antibodies and dilutions were optimized for the following targets: GLUT1 (EMD Millipore; 1:1000), GLUT3 (Protein Tech; 1:2000), hexokinase-1 (Cell Signaling; 1:2000), caveolin-1 (Cell Signaling; 1:1000), MCT1 (EMD Millipore; 1:1000), MCT2 (Santa Cruz; 1:1500), MCT4 (Protein Tech; 1:1500), AACS (EMD Millipore; 1:1000), SCOT (Protein Tech; 1:3000), a cocktail of 5 OXPHOS subunits (Abcam; 1:5000), apoE (EMD Millipore; 1:1000), and α-tubulin (Cell Signaling; anti-rabbit 1:2000; anti-mouse 1:150,000). Secondary antibodies consisted of goat anti-mouse IgG-HRP (Santa Cruz), goat anti-rabbit IgG-HRP (Santa Cruz), donkey anti-goat IgG-HRP (Santa Cruz), goat anti-rabbit DyLight 800 (Thermo Scientific), and goat anti-mouse DyLight 680 (Thermo Scientific).

Protein data was collected across triplicate western blots and was first normalized to the average signal per blot; each lane was corrected by normalized α-tubulin. The triplicate results for each subject were then averaged. Each blot contained equal numbers of *APOE4* carrier and non-carrier samples. Statistical analysis was conducted using 2-tailed unpaired Student's *t*-tests, following Levene's test for equal variances and NPP plot inspection for normality, with the significance level corrected from $p < 0.05$ for multiple comparisons with the Benjamini-Hochberg procedure to control the false discovery rate (spreadsheet from Manuel Weinkauf, MARUM, https://marum.de/software_and_programs.html) [37]. Statistical analyses were performed in IBM SPSS or Microsoft Excel.

For RNA quantitation, small samples were cut from each frozen tissue block, quickly weighed and placed in RNase-free microcentrifuge tubes. Samples were analyzed using either an array platform based on quantitative real time PCR (qRT-PCR) or by using individual pre-validated qRT-PCR primers. RNA was extracted using TRIZOL reagent (Invitrogen) followed by purification via the Qiagen RNeasy kit. An on-column DNase treatment was used to remove residual genomic DNA and absence of genomic DNA was confirmed prior to use. cDNA was synthesized from 1 μg total RNA using SA Biosciences RT2 First-Strand Kit. Array platform gene expression profiling was then performed using the RT2 Profiler PCR Array System (SA Biosciences) which analyzes a panel of 84 genes in related biological pathways using reliable SYBR Green-optimized primer assays. Array analysis was performed using the Human Mitochondrial Energy Metabolism Array (PAHS-008YC), which primarily profiles genes involved in the electron transport chain. The expression of additional genes was analyzed by qRT-PCR using individual pre-validated human primers from SA Biosciences (GLUT1, GLUT3, Hexokinase1, Caveolin1, MCT1, MCT2, MCT4). Experiments used an ABI StepOne Plus qRT-PCR machine. Ct thresholds were manually set within the linear phase of the logarithmic amplification plots using the StepOne Plus software. Data from logarithmic plots was analyzed using the downloadable Excel PCR Array Data Analysis Template v.4.0 by SA Biosciences (GeneGlobe Data Analysis Center). This template uses the $\Delta\Delta$Ct method to establish the fold change between control and experimental groups. The template also employs

a two-tailed Student's *t*-test to establish statistical significance using $p < 0.05$. A set of housekeeping genes (Gapdh, Actb, and RPLP0) which are included on the array plates, was used for normalization of qRT-PCR array data by subtracting the average Ct value of the housekeeping genes in each sample from the Ct values of the genes of interest in that sample. The expression of genes measured by individual qRT-PCR primer analysis was normalized using the beta-2 microglobulin housekeeping gene.

RESULTS

The non-carrier subjects (7 males, 5 females; 9 Caucasian, 2 African-American, 1 Hispanic) were made up of 11 *APOE* 3/3 homozygotes and 1 *APOE* 2/3 heterozygote; the carrier subjects (6 males, 7 females; 8 Caucasian, 5 African-American) consisted of 11 *APOE* 3/4 heterozygotes and 2 *APOE* 4/4 homozygotes (Table 1). None of the *APOE* 2/3 or *APOE* 4/4 subjects displayed protein results that stood apart within their respective groups; however, they did increase variability within the qPCR analysis and thus the final qPCR results reflect only *APOE* 3/3 and *APOE* 3/4 subjects. One *APOE* 3/3 subject and one *APOE* 3/4 subject were removed from western blot analysis due to abnormal α-tubulin signal and insufficient tissue available to run all blots, respectively. Between APOE4 non-carrier and carrier groups there were no significant differences in age (non-carriers: 27.4 ± 6.0y, carriers: 31.7 ± 6.5 [mean ± standard deviation]; Student's *t*-test, $p = 0.112$). There were also no significant differences in postmortem interval (non-carriers: 13.3 ± 4.2h, carriers: 16.5 ± 4.1 [mean ± standard deviation]; Student's *t*-test, $p = 0.065$).

This subset of subjects did display group differences in mitochondrial cytochrome oxidase function, from data included in our previous analysis [28], indicating a reduction in oxidative phosphorylation capacity. Specifically, this prior analysis utilized quantitative histochemistry on PCC slices in order to measure the enzymatic activity of cytochrome c oxidase (Complex IV) of the electron transport chain [32]. As in the previous analysis, cortical lamina layer I activity was significantly decreased in this subset of the same *APOE4* carrier subjects (non-carriers: 372.0 ± 25.8, carriers 340.9 ± 15.4 [mean ± standard deviation]; Student's *t*-test, $p < 0.001$), as well as in layer II (non-carriers: 373.2 ± 13.0, carriers 348.5 ± 11.5; Student's *t*-test, $p < 0.001$), and layer III (non-carriers: 379.3 ± 14.6, carriers 358.0 ± 12.9; Student's *t*-test, $p < 0.001$). Notably, these subjects were also previously shown to lack any group differences in pathology markers including from ELISA (soluble $A\beta_{1\text{-}40}$, insoluble $A\beta_{1\text{-}40}$, and soluble $A\beta_{1\text{-}42}$, insoluble $A\beta_{1\text{-}42}$) or Aβ Immunohistochemistry, Thioflavin S, AT8 Immunohistochemistry, or Campbell-Switzer analyses (data not shown) [28]. The relative levels of apoE were quantified and no differences were found (non-carriers: 106.5 ± 51.7; carriers: 101.7 ± 22.2 [normalized mean ± SEM], Student's *t*-test, $p = 0.82$).

Overall, despite manifesting a significant *decline* in cortical energy metabolism, these young *APOE4* carriers displayed significant *increases* in the apparent expression of many of the key proteins underlying energy metabolism (Fig. 1). Since the cerebral metabolic rate for glucose (CMRglu) has been

Table 1
Demographics of analyzed subjects

APOE	Sex	Age (y)	PMI (h)	Race	COD
ε4 non-carriers					
3/3	M	29	12	H	MVA
3/3	M	19	11	C	narcotic intoxication
3/3	M	35	12	C	MVA
3/3	F	33	19	C	ASCVD
3/3	M	23	8	AA	cardiomyopathy
3/3	F	20	19	C	MVA
2/3	F	18	15	C	MVA
3/3	F	26	12	C	cardiac tamponade
3/3	M	30	20	C	MVA
3/3	M	33	7	AA	coronary thrombosis
3/3	M	31	13	C	MVA
3/3	F	32	12	C	MVA
ε4 carriers					
3/4	F	39	17	C	MVA
3/4	M	24	8	C	compressional asphyxia
3/4	M	35	20	C	ASCVD
3/4	F	33	20	AA	MVA
3/4	M	28	17	C	MVA
3/4	M	37	12	AA	ASCVD
3/4	M	21	13	AA	drowning
4/4	F	34	12	AA	asthma
3/4	F	21	22	AA	asthma
4/4	F	27	18	C	gunshot wound
3/4	F	33	17	C	asthma
3/4	M	40	20	C	HASCVD
3/4	F	40	19	C	narcotic intoxication

PMI, postmortem interval; AA, African American; C, Caucasian; H, Hispanic; ASCVD, arteriosclerotic cardiovascular disease; HASCVD, hypertensive arteriosclerotic cardiovascular disease; MVA, motor vehicle accident.

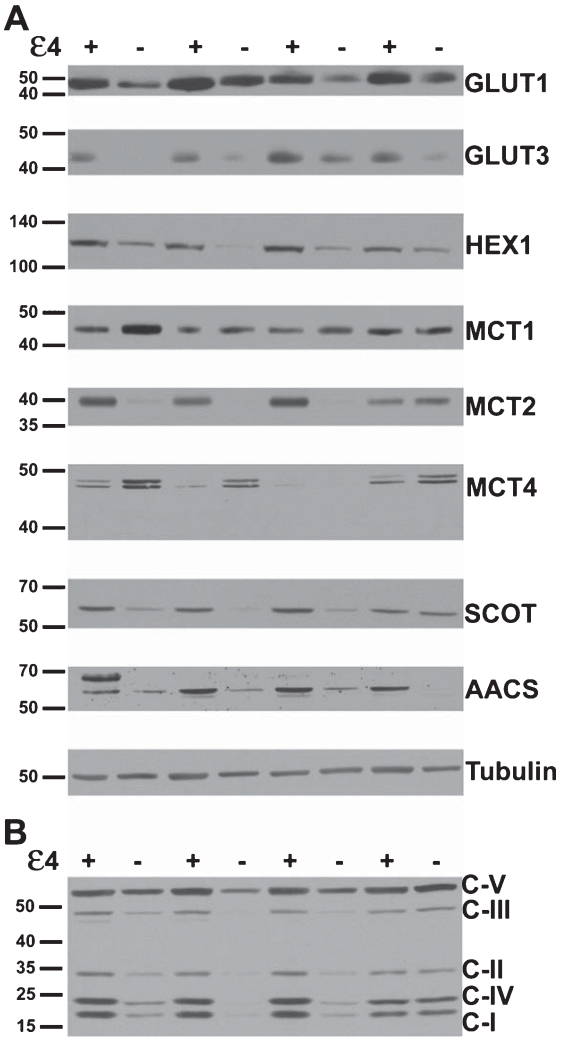
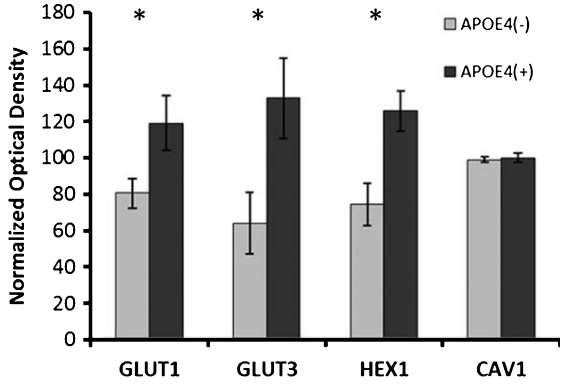


Fig. 1. Several key metabolic proteins show altered expression in *APOE4* carriers. A) Representative western blots of proteins underlying brain glucose and ketone metabolism. Each protein target was analyzed with a separate probe; however, GLUT1, GLUT3, MCT1, and MCT4 results shown here align to the same subjects. Similarly, HEX1, MCT2, SCOT, and AACS shown here align to the same second set of subjects. Analysis proceeded on three such sets in total for each target (N = 24), in triplicate. B) A representative western blot resulting from application of the antibody cocktail against mitochondrial oxidative phosphorylation protein subunits. Analysis proceeded on three such sets (N = 24), in triplicate.

shown to be decreased in similarly-aged young adult *APOE4* carriers [20], we assessed protein levels and RNA expression for glucose transport (GLUT1, GLUT3), metabolism (hexokinase), and associated membrane domains (caveolin). Results are shown in Fig. 2. GLUT1 (non-carriers: 80.5 ± 7.9, carriers: 119.1 ± 16.9 [normalized mean $\pm$ SEM]; Student's *t*-test, $p = 0.035$), GLUT3 (non-carriers: 64.2 ± 16.7, carriers: 132.9 ± 23.2; Student's *t*-test, $p = 0.022$), and hexokinase-1 (non-carriers: 74.5 ± 16.7, carriers: 125.5 ± 11.4; Student's *t*-test, $p = 0.004$), but not caveolin (non-carriers: 98.9 ± 1.51, carriers: 99.9 ± 2.7; Student's *t*-test, $p = 0.757$), were significantly altered in the APOE4 carriers. Quantification of total mRNA validated the changes in GLUT3 (1.65x; Student's *t*-test, $p = 0.024$) and hexokinase-1 (1.64x; Student's *t*-test, $p = 0.007$), matched the lack of significant change in caveolin (1.04x; Student's *t*-test, $p = 0.790$), but did not correlate with the increase in GLUT1 protein (1.1x; Student's *t*-test, $p = 0.79$).

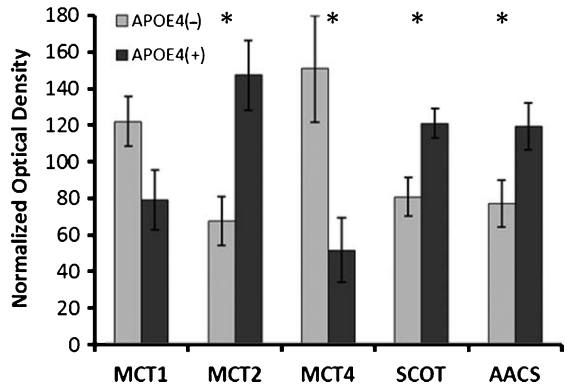
Considering the observation that *APOE4*-positive AD patients do not appear to benefit from ketogenic therapies [38, 39], we assessed protein and RNA expression for monocarboxylate (ketone/lactate/pyruvate) transport and ketone metabolism. Results are shown in Fig. 3. MCT2 (non-carriers: 67.7 ± 13.7, carriers: 147.3 ± 19.9 [normalized mean $\pm$ SEM], Student's *t*-test, $p = 0.003$), ketolytic enzyme succinyl-CoA:3-ketoacid CoA transferase (SCOT) (non-carriers: 80.9 ± 10.6, carriers: 121.1 ± 8.2, Student's *t*-test, $p = 0.006$), and ketone-utilizing acetoacetyl-CoA synthetase (AACS) (non-carriers: 77.2 ± 13.1, carriers: 119.5 ± 13.4, Student's *t*-test, $p = 0.031$) showed altered protein levels. Also evaluated for altered protein levels were MCT1 (non-carriers: 122.1 ± 13.6, carriers: 79.2 ± 17.0 [normalized mean $\pm$ SEM], Student's *t*-test, $p = 0.058$) and MCT4 (non-carriers: 150.9 ± 29.3, carriers: 51.7 ± 18.6, Student's *t*-test, $p = 0.008$), which are monocarboxylate transporters on astroglia and endothelial cells. In the mRNA quantification (applied only to the transporters), the increase in MCT2 was validated (1.55x; Student's *t*-test, $p = 0.008$), and the lack of change in MCT1 was similarly validated (1.02x; Student's *t*-test, $p = 0.85$), but the decline in MCT4 was not matched by the mRNA quantification (1.03x; Student's *t*-test, $p = 0.74$).

Since our previous results in these *APOE4*-positive subjects indicated a decline in oxidative phosphorylation capacity, we assessed select OXPHOS protein subunits using a cocktail of antibodies targeting a subunit from each of the five OXPHOS complexes. Results are shown in Fig. 4. Multiple subunit proteins showed increased expression, including Complex I (non-carriers: 59.2 ± 15.1, carriers: $146.4.3 \pm 31.6$ [normalized mean $\pm$ SEM], Student's *t*-test, $p = 0.020$), Complex II (non-carriers: 63.9 ± 12.6, carriers: 137.3 ± 26.1, Student's *t*-test, $p = 0.018$), and Complex IV (non-carriers: 55.6 ± 15.2, carriers: 147.4 ± 32.1, Student's *t*-test, $p = 0.017$).



mRNA	Fold Change Test Sample/Control Sample	*t*-test p value
GLUT1	1.1	0.793
GLUT3	1.64	*0.024*
Hexokinase-1	1.65	*0.007*
Caveolin-1	1.04	0.788

Fig. 2. Alterations in glucose metabolism in *APOE4* carriers. Western blot results demonstrating significantly higher protein levels of glucose metabolism proteins in *APOE4* carriers, except CAV1 (normalized mean ± SEM). mRNA transcripts for GLUT3 and hexokinase-1 were shown to be increased in qPCR analysis (table right); GLUT1 and caveolin-1 were not significantly higher in the qPCR analysis. GLUT1, GLUT3, glucose transporters; HEX1, hexokinase-1; CAV1, caveolin-1. APOE4(+), carriers; APOE4(–), non-carriers. * $p < 0.0375$ (Benjamini-Hochberg adjusted significance level), 2-tailed Student's *t*-test.



mRNA	Fold Change Test Sample/Control Sample	*t*-test p value
MCT1	1.02	0.854
MCT2	1.55	*0.008*
MCT4	1.03	0.743

Fig. 3. Alterations in ketone metabolism in *APOE4* carriers. Western blot results demonstrating significantly altered protein levels for ketone metabolism in *APOE4* carriers (normalized mean ± SEM). MCT2, SCOT, and AACS were significantly increased, and MCT4 was significantly decreased. MCT2 mRNA transcript increases were demonstrated via qPCR. MCT1, MCT2, MCT 4, monocarboxylate transporters; SCOT, succinyl-CoA:3-ketoacid CoA transferase; AACS, acetoacetyl CoA synthetase; APOE4(+), carriers; APOE4(–), non-carriers. * $p < 0.040$ (Benjamini-Hochberg adjusted significance level), 2-tailed Student's *t*-test.

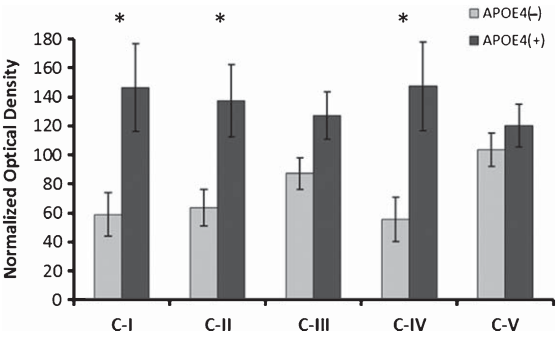


Fig. 4. Alterations in electron transport chain protein expression in *APOE4* carriers. Western blot results demonstrating significantly increased expression of ETC complex I, II and IV subunits in *APOE4* carriers (normalized mean ± SEM). CI-CV, ETC Complexes I-V, respectively; APOE4(+), carriers; APOE4(–), non-carriers. * $p < 0.030$ (Benjamini-Hochberg adjusted significance level), 2-tailed Student's *t*-test.

Complex III (non-carriers: 87.3 ± 10.7, carriers: 127.1 ± 17.3, Student's *t*-test, $p = 0.057$) and Complex V were not significantly altered (non-carriers: 103.4 ± 11.5, carriers: 120.1 ± 15.5, Student's *t*-test, $p = 0.384$). The Mitochondrial Energy Metabolism qPCR array demonstrated only 3 (of 84) significant gene expression changes, each with small fold-changes (*NDUFB5* 1.08x, Student's *t*-test, $p = 0.048$; *NDUFS7* -1.18x, Student's *t*-test, $p = 0.048$; *ARRDC3* -1.39x, Student's *t*-test, $p = 0.018$). It should be noted that the Complex IV subunit is encoded within mitochondrial DNA; the others are encoded in nuclear DNA.

DISCUSSION

APOE4 is strongly associated with an elevated risk for late-onset AD, but how this increased risk is

mediated is not well understood. In brain imaging and histochemical studies, *APOE4* carriers consistently demonstrate reductions (when compared to *APOE4* non-carriers) in energy metabolism via measures of mitochondrial function and glucose metabolism, even decades before possible disease onset [20, 28] and in a pattern consistent with findings in AD patients [19, 31, 40]. Here, we report that these findings are accompanied by underlying alterations in the PCC, a key region in AD-associated functional brain changes, in components of energy metabolism in a set of young adult *APOE4* carriers previously demonstrated to have declines in mitochondrial function in the absence of apparent amyloid or tau pathology [28]. Our focus here is on details of altered function and potential proximal causes explainable by cell biology. We do not argue that these findings are necessarily indicative of a primary defect in energy metabolism, although in some cases this is possible. Fundamentally, these findings may represent alterations or compensation in metabolic processes as a result of the altered synaptic structure [41–43] and function [30] that may exist in *APOE4* carriers.

In this study, we examined GLUT1 (primary blood-brain barrier glucose transporter) and GLUT3 (neuronal glucose transporter), key proteins in glucose metabolism. In the context of AD, GLUT1 reductions have been found in AD patients [44] and GLUT1 deficiency and *APOE4* have been linked to an exacerbation of amyloid induced pathophysiological effects on the blood-brain barrier in mice [45–47]. A study of AD patients with reduced protein levels of GLUT1 indicated that mRNA levels were not impacted, pointing to possible alterations in post-transcriptional regulation [48]. Here we demonstrate *increased* protein levels for GLUT1 in the absence of changes in mRNA levels, again indicating regulation by a potential shift in translational activity. Studies in aged *APOE4* targeted replacement mice, in which the human protein is expressed under the control of the endogenous *APOE* promoter [49] have found that these mice display decrements in glucose transport across the blood-brain barrier, but do not have altered levels of GLUT1 protein or mRNA [50]. Further investigation will be needed to resolve these discrepancies, which may arise from the specific patterns and timing of blood-brain barrier effects, or from differences in GLUT1 post-transcriptional regulation [51]. Regarding GLUT3 (neuronal glucose transporter), we found increased levels of protein accompanied by increased mRNA. AD patients have been shown to have reduced levels of GLUT3 protein [44]. It is possible in *APOE4* young adults that this increase in expression is a compensatory response to defective trafficking of GLUT3 to the surface membrane of the neuron. GLUT3 trafficking in neurons is known to be regulated by synaptic activity [52]; perhaps the aberrant synaptic activity associated with *APOE4* plays a role in this process leading to more protein being produced as less protein is functional (i.e., less is localized at the surface membrane). Interestingly, apoE binds rab11, a protein involved in the surface trafficking of GLUT3, which may modulate or interfere with the transporter trafficking process [53, 54]. While we might expect protein levels to be down in a pattern more intuitively consistent with hypometabolism (and consistent with AD patients), mechanisms effecting functional distribution of protein are equally important and may lead to compensatory changes. However, comparison with AD patients is often complex, as it is often unclear if findings in AD patients are the result of a disrupted physiological process (earlier disease) or simply evidence of gross degradation (later disease). Notably, hexokinase-1 (which functions to sequester imported glucose in cells via phosphorylation) protein levels were also elevated in *APOE4* carriers, possibly as a compensatory response to changes in functional glucose transport. Overall, it is also possible that increases in proteins involved in glucose metabolism are reflective of the dysregulation of peripheral metabolism that is a feature of *APOE4* mouse models [55]. Due to the importance of finely tuned energy metabolism processes for proper synaptic function [34], any defects in young adults could play an important role in AD risk.

In addition to these alterations in the metabolism of glucose, we detected altered levels of two key proteins involved in monocarboxylate transport, MCT2 and MCT4, which transport lactate and ketones across the blood-brain barrier and astrocytic membranes [56]. Ketone metabolism has been linked to AD pathophysiology [57], and ketone therapies have been associated with therapeutic benefit in small clinical studies [38, 39]. While ketone therapies have a long history of effectiveness in treating forms of epilepsy, the mechanisms underlying neurologic benefit remain under active investigation [58]. Since *APOE4* AD patients do not appear to show cognitive benefit in response to ketogenic therapy that are observed in other AD patients [39], the apparent effects on MCT4 may be an underlying cellular-level mechanism for this discrepancy. Notably, MCT4 protein levels were lower in *APOE4* carriers, while other

metabolic proteins were increased. Of note, endoplasmic reticulum stress (e.g., unfolded protein response) has been found in astrocyte cultures as a result of apoE4 and has been shown to impair receptor processing [59, 60]. Such a mechanism could account for the decreased MCT4 levels demonstrated in the present study. Interestingly, MCT2, the primary neuronal transporter, was found to have increased protein and mRNA levels. While, to our knowledge, MCT2 has not been assessed in AD patients, MCT2 protein levels are known to be decreased in Aβ-treated rats [61]. This is a pattern consistent with our findings regarding glucose metabolism, as detailed above. MCTs also transport lactate, a known neuroprotectant [62], in addition to ketones, revealing an additional risk mechanism with alterations in this energy metabolism pathway. Notably, enzymes involved in ketone metabolism (AACS and SCOT) also showed increases in protein levels indicating additional alterations in this pathway associated with the expression of apoE4. Similar to the protein expression changes in the glucose pathway, these changes in monocarboxylate transport may also be linked to peripheral metabolic dysregulation.

Mitochondrial dysfunction has long been linked to AD pathophysiology [63–69]. Here, mitochondrial oxidative metabolism is altered in young adult *APOE4* carriers in a manner that is opposite to what is seen in the disease state. We and others have previously reported diminished brain cytochrome oxidase activity in AD patients and in these same young adult subjects [28, 31]. In this study we demonstrate increased protein levels in complexes I, II, and IV of the ETC in carriers. However, in AD, using nearly identical techniques, we demonstrated significantly lower expression of these ETC proteins in the same brain region [70]. Our qRT-PCR array analysis of genes involved in mitochondrial energy metabolism failed to identify altered transcriptional expression of the ETC subunit genes, indicating that the *APOE4* allele is not directly affecting transcription. ApoE4 has been linked to ETC dysfunction via binding of cleaved fragments to ETC subunits causing a decrease in functional activity [71]. Hence, it is possible that this is the mechanism underlying the increases in protein levels (perhaps compensatory as discussed above) and the decreased functional activity found in prior histochemical studies. A prior study of postmortem and biopsied tissues from AD patients also demonstrated deficits in the quantity and quality of mitochondria, with cytochrome c oxidase subunit I protein levels and mtDNA increased several fold in comparison to controls, with high brain-regional specificity and apparent localization in autophagosomes [67]. This is suggestive of mitochondrial protein levels being upregulated as a compensatory response to dysfunction, potentially in a manner similar to what may be occurring in *APOE4* carriers decades before possible AD onset. Again, mitochondria are a fundamental player in neural energy metabolism, and any alteration in these physiological processes may impair synaptic function [66]. *TOMM40*, a gene encoding Tom40, a protein involved in transporting proteins into the mitochondria, has been proposed as a genetic risk factor for AD, although the extent to which individual variants of *TOMM40* impact AD risk is not yet clear [72–76]. Notably, *TOMM40* and *APOE* are in linkage disequilibrium on chromosome 19, and due to this fact, parsing out the respective contributions of these genes is complex [72]. It is possible that altered Tom40 function causes a defect in protein import into the mitochondria and leads to increased protein levels due to buildup in the cytoplasm. Notably, many ETC proteins subunits are encoded in the nuclear (not mitochondrial) genome. Further studies incorporating *TOMM40* status may play an important role in examining this intriguing possibility.

This study has multiple strengths. First, to our knowledge this is the first study to attempt to analyze energy metabolism pathways in the at-risk *APOE4* population in order to characterize some of the physiological alterations underlying the well-established finding of hypometabolism. Here we present detailed findings of different pathways, several of which have previously been associated with AD, but have been unexplored in *APOE4* subjects. Second, we focus on the PCC, a highly metabolically-active brain region that is a functional hub for brain networks that are disrupted in AD, indicating that it may show altered function early in AD risk-associated processes [23, 27, 77–79]. Third, our analysis was done in young adults, who as *APOE4* carriers demonstrate a vulnerability to AD, but who are also decades from possible disease onset and free from apparent AD-related pathology.

There are several opportunities for future investigation that arise from this study. The subjects presented here were selected from an existing, previously analyzed pool, and thus the *APOE4* carriers were known to show functional metabolic decline *a priori*. Thus, these findings should be replicated in a larger and independent group of subjects. Additionally, while we have identified alterations in metabolic pathways

in *APOE4* carriers, the mechanics of how these alterations occur remain unclear (above). While *APOE4* has been linked to altered dendritic complexity [41] and modification of synaptic plasticity [30] that may be associated with these functional findings, the effects of these structural factors, in addition to potential primary energy defects, are difficult to parse in human subjects. Further investigation in robust experimental models would not only allow for the confirmation of our results, but for a more complete understanding of how *APOE4*-induced changes in metabolism affect the pathogenesis of, and potential resistance to, AD. In this context, efficient metabolism may serve as a buffer (i.e., reserve) to help maintain function in the face of AD-related changes [80, 81]. Further exploring the link between metabolism and synaptic function, and how this relationship is modulated by *APOE4* will also be of fundamental importance [82]. It is also possible that alterations in metabolism are reflective of some AD-related pathophysiological process not associated with *APOE4* or that metabolic changes are a readout of risk, but not a risk factor per se. In this case, further understanding of metabolic changes may lead to the development of more specific and sensitive biomarkers; some specific metabolic pathways that become altered may be more appropriate than looking at FDG PET alone. For instance, new imaging tools examining aerobic glycolysis have demonstrated interesting patterns of glucose utilization in the brain, but to our knowledge these tools have not been used in longitudinal examination of at-risk populations [24, 26]. A point to consider is that we did not approach this survey of energy metabolism from an unbiased perspective, as we selected specific pathways with prior evidence of their involvement in AD. Ongoing work utilizing next-generation techniques such as RNA-seq with cell-type specificity [83] is addressing this issue and providing a valuable alternative approach.

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