



Using lipid profiling to better characterize metabolic differences in *apolipoprotein E (APOE)* genotype among community-dwelling older Black men

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Abstract *Apolipoprotein E (APOE)* allelic variation is associated with differences in overall circulating lipids and risks of major health outcomes. Lipid profiling provides the opportunity for a more detailed description of lipids that differ by *APOE*, to potentially inform therapeutic targets for mitigating higher morbidity and mortality associated with certain *APOE* genotypes. Here, we sought to identify lipids, lipid-like molecules, and important mediators of fatty acid metabolism that differ by *APOE* among 278 Black men ages 70–81. Using liquid chromatography-mass spectrometry methods, 222 plasma metabolites classified as lipids, lipid-like molecules, or essential in fatty acid metabolism were detected. We applied

principal factor analyses to calculate a factor score for each main lipid category. *APOE* was categorized as $\epsilon 4$ carriers ($n=83$; $\epsilon 3\epsilon 4$ or $\epsilon 4\epsilon 4$), $\epsilon 2$ carriers ($n=58$; $\epsilon 2\epsilon 3$ or $\epsilon 2\epsilon 2$), or $\epsilon 3$ homozygotes ($n=137$; $\epsilon 3\epsilon 3$). Using analysis of variance, the monoacylglycerol factor, cholesterol ester factor, the factor for triacylglycerols that consist mostly of polyunsaturated fatty acids, sphingosine, and free carnitine significantly differed by *APOE* ($p < 0.05$, false discovery rate < 0.30). The monoacylglycerol factor, cholesterol ester factor, and sphingosine were lower, whereas the factor for triacylglycerols that consisted mostly of polyunsaturated fatty acids was higher among $\epsilon 2$ carriers than remaining participants. Free carnitine was lower among $\epsilon 4$ carriers than $\epsilon 3$ homozygotes. Lower monoacylglycerols and cholesteryl esters and higher triacylglycerols that consist mostly of polyunsaturated fatty acids may be protective metabolic characteristics of *APOE* $\epsilon 2$ carriers, whereas lower carnitine may reflect altered mitochondrial functioning among $\epsilon 4$ carriers in this cohort of older Black men.

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Introduction

The *apolipoprotein E (APOE)* gene has three common alleles: $\epsilon 2$, $\epsilon 3$, and $\epsilon 4$. *APOE* encodes for the glycoprotein, apolipoprotein E, which combines

with lipids to form lipoproteins that transport lipids through the bloodstream to different tissues for energy or storage [1]. *APOE* allelic variation causes apolipoprotein E to differ in lipid preferential binding; the $\epsilon 4$ allele causes apolipoprotein E to have a higher affinity for larger very low-density lipoprotein (VLDL) particles rich in triglycerides [2], whereas $\epsilon 2$ and $\epsilon 3$ alleles cause apolipoprotein E to have a higher affinity for smaller high-density lipoprotein (HDL) particles rich in phospholipids [2]. Another important distinction is the $\epsilon 2$ allele causes apolipoprotein E to have a 50 times lower affinity for binding to low-density lipoproteins (LDL) than the $\epsilon 4$ and $\epsilon 3$ alleles [3].

Differences in lipoprotein affinity by *APOE* result in differences in lipid metabolism and circulating lipids [2]. For example, the enhanced binding of apolipoprotein E to VLDL particles due to the $\epsilon 4$ allele results in altered lipid metabolism [4], characterized by increased liver uptake of LDL particles that paradoxically results in increased circulating LDL particles [5]. Specifically, $\epsilon 4$ carriers have higher triglycerides and LDL cholesterol, whereas $\epsilon 2$ carriers have higher triglycerides, but lower LDL cholesterol than $\epsilon 3$ homozygotes [6]. *APOE* $\epsilon 4$ carriers also have higher risks of Alzheimer's disease [7, 8], cardiovascular disease [9, 10], and mortality [11], whereas $\epsilon 2$ carriers have lower risks of Alzheimer's disease [12], cardiovascular disease [10], and mortality [11] when compared to $\epsilon 3$ homozygotes. U.S. frequencies for the $\epsilon 2$, $\epsilon 3$, and $\epsilon 4$ alleles are 0.07, 0.78, and 0.15, respectively [13], with a higher frequency of the $\epsilon 4$ "risk" allele among Black versus White older adults [14–18]. A more detailed description of lipids that differ by *APOE* could potentially inform novel therapeutic targets to mitigate morbidity and mortality related to *APOE*.

Metabolomics is the large-scale study of small molecules that are intermediates or end-products of cellular metabolism and typically include numerous lipids and lipid-like molecules. The Health, Aging, and Body Composition (Health ABC) study measured 350 plasma metabolites in a subset of 319 older Black men, of which more than 200 were classified as lipids or lipid-like molecules. Here, we sought to better characterize known differences in overall circulating lipids according to *APOE* by identifying lipids, lipid-like molecules, and important mediators of fatty acid metabolism that differ

among older Black men who carry the *APOE* $\epsilon 4$ allele ($n = 83$; $\epsilon 3\epsilon 4$, $\epsilon 4\epsilon 4$), $\epsilon 2$ allele ($n = 58$; $\epsilon 2\epsilon 3$, $\epsilon 2\epsilon 2$), or two copies of the $\epsilon 3$ allele ($n = 137$; $\epsilon 3\epsilon 3$).

Methods

Cohort

The Health ABC study was a prospective cohort of 3075 Black and White men and women [19]. Participants were recruited from Pittsburgh, Pennsylvania and Memphis, Tennessee. White individuals were recruited from a random sample of Medicare beneficiaries and all age-eligible Black individuals were recruited. Eligible participants were ages 70–79 during recruitment (March 1997–July 1998) and self-reported no difficulty walking $\frac{1}{4}$ mile, climbing ten steps, or with basic activities of daily living. Ineligibility criteria included history of active cancer within 3 years or planning to move from the study area during the next 3 years. The study was approved by each sites' Institutional Review Board. All participants provided written informed consent. All procedures were in accordance with institutional guidelines.

In 2016, a pilot study detected 350 metabolites in plasma collected at year 2 (1998–99) after an overnight fast of at least 8 h among a subset of 319 Black men, to better understand the influence of body composition on cellular metabolism [20]. The study was restricted to Black men due to limited funds and because U.S. Black individuals have a higher prevalence of aging-related metabolic diseases, but more muscle mass than U.S. White individuals [20]. The study was restricted to men to limit heterogeneity in body composition due to differences by sex.

APOE

Genotyping was performed at year 1 by the Center for Inherited Disease Research using Illumina Human1M-Duo BeadChip v3.3.7 system [21]. *APOE* was determined directly using polymerase chain reaction on coded DNA samples [11]. For analyses, *APOE* was categorized as $\epsilon 4$ carriers ($\epsilon 3\epsilon 4$ and $\epsilon 4\epsilon 4$; $n = 83$), $\epsilon 2$ carriers ($\epsilon 2\epsilon 3$ and $\epsilon 2\epsilon 2$; $n = 58$), or $\epsilon 3$ homozygotes ($n = 137$). Fourteen participants carrying the $\epsilon 2\epsilon 4$ alleles were examined but excluded from statistical tests due to the small number of

participants. The proportions of *APOE* categories among the subset of Black men with metabolomics were almost identical to the proportions among the total sample of Health ABC Black men (Supplemental Table 1a versus 1b). The Health ABC Black men had higher proportions of $\epsilon 2$ carriers and $\epsilon 4$ carriers and a lower proportion of $\epsilon 3$ homozygotes than the White participants (Supplemental Table 1b).

Metabolites

Metabolites were measured by the Broad Institute using plasma extracts collected at year 2 after an overnight fast of at least 8 h. These plasma samples had never been thawed and were stored at $-80\text{ }^{\circ}\text{C}$ from collection (1998/1999) until metabolite profiling (2016). Three liquid chromatography-mass spectrometry (LC-MS) metabolite profiling methods were previously described in detail [22–24], measuring: (1) amines and polar metabolites; (2) central metabolites and polar metabolites, and (3) lipids [23]. Metabolite values were analyzed using TraceFinder (ThermoFisher Scientific, Waltham, MA, USA) and Progenesis QI (Nonlinear Dynamics, UK), confirmed manually using known standards, and if below the limit of quantitation (signal/noise < 10) were classified as unquantifiable [22]. Two pooled samples were run after every 20 samples, one to normalize the data, if necessary, and the other to assess quality of that normalization. The positive and negative ion mode detection methods used normalization to the nearest neighbor, whereas the lipid profiling method was not normalized. We found high reliability of known metabolites from sixteen blinded duplicates (intra-class correlation coefficient, median: 0.92; interquartile range: 0.81–0.97) [20]. Metabolite values were reported as LC-MS peak areas, which are proportional to concentrations [20, 23]. Metabolites were log-transformed and standardized to a mean of zero and standard deviation of one.

A total of 350 metabolites were detected using the three profiling methods [20]. For this report, our independent variable of interest, i.e., *APOE*, was a three-level categorical variable with a limited sample size, thus, to reduce the number of comparisons made, we focused on a subset of the 350 metabolites (Supplemental Table 2). We examined metabolites classified as lipids (e.g., triacylglycerols, cholesteryl

esters, and sphingomyelins) or lipid-like molecules (bile acids, acylcarnitines) and metabolites that play a central role in fatty acid metabolism (e.g., free carnitine). Specifically, we examined a subset of 229 metabolites. Among the 229 metabolites, 189 were detected using the lipid profiling method, eight were detected using the polar metabolite profiling method with negative ion mode detection (i.e., five bile acids, two fatty acids, and one glycerophosphate), and 32 were detected using the polar metabolite profiling method with positive ion mode detection (i.e., 28 acylcarnitine, free carnitine, two fatty acids, and one glycerophosphocholine).

Among the subset of 229 metabolites, 206 were detected in all participants. Sixteen metabolites were detected in $\geq 80\%$ of the cohort; missing values were assumed to be due to true values being below detectable limits [25] and for analyses, these missing values were replaced with half the minimum recorded value for the respective metabolite [25]. The remaining seven metabolites were excluded from analyses because they were detected in < 80% of participants [26]. Thus, our final analysis examined 222 metabolites by *APOE* (Supplemental Table 3).

Participant characteristics

Participants self-reported age, gender, Black race, highest level of education, and cigarette smoking habits. Height, weight, and waist circumference were recorded and body mass index was calculated. Usual diet during the past year was estimated using an interviewer-administered 108-item Food Frequency Questionnaire [27]. Using total body dual-energy x-ray absorptiometry (Hologic QDR 4500A; Hologic, Bedford, MA, USA), appendicular lean mass was estimated as the sum of bone-free lean mass in arms and legs divided by height² and body fat was estimated relative to total mass. History or presence of cardiovascular disease, hypertension, diabetes, and cancer were based on self-report of a physician diagnosis or taking medication. Participants brought in all prescription medications used in the last 2 weeks for an inventory, and information on use of statins or other lipid-lowering medications was documented. Serum interleukin-6 and C-reactive protein and plasma total cholesterol were measured at year 2 by a core laboratory at Wake Forest University. Serum

Table 1 Characteristics^a of Health ABC Black men by *APOE* genotype categories

Mean (standard deviation) or frequency (percent)	<i>APOE</i> genotype categories				Overall <i>p</i> -value ^b ; pairwise comparisons ^c
	$\epsilon 2$ carriers (<i>n</i> = 58)	$\epsilon 3$ homozygotes (<i>n</i> = 137)	$\epsilon 4$ carriers (<i>n</i> = 83)	$\epsilon 2\epsilon 4$ carriers (<i>n</i> = 14)	
Age	75 (2.5)	75 (2.9)	75 (2.9)	73 (2.1)	.99
Pittsburgh site	31 (53%)	77 (56%)	47 (57%)	6 (43%)	.92
More than high school education	9 (16%)	44 (32%)	25 (30%)	3 (21%)	.06
Current smoker, year 1	10 (17%)	23 (17%)	17 (20%)	5 (36%)	.78
Height (cm), year 1	172 (7.2)	174 (6.3)	172 (7.5)	171 (6.3)	.16
Weight (kg)	78 (15)	83 (14)	79 (15)	80 (12)	.05
Body mass index (kg/m ²)	26 (4.6)	27 (4.5)	27 (4.4)	28 (3.8)	.18
Waist circumference (cm), year 1	98 (13)	101 (11)	98 (13)	98 (11)	.18
Appendicular lean mass (kg/m ²)	8.2 (1.3)	8.4 (1.1)	8.2 (1.1)	8.4 (1.1)	.32
Body fat (%)	27 (5.4)	29 (5.6)	28 (5.4)	29 (6.3)	.25
Daily dietary intake:					
Total calories (Kcal)	2239 (1355)	2145 (900)	2309 (1168)	2375 (1448)	.55
Protein kilocalories (%)	13 (2.7)	14 (3.1)	14 (3.6)	16 (2.7)	.13
Fat kilocalories (%)	36 (7.6)	35 (7.6)	34 (7.0)	35 (7.8)	.50
Carbohydrates kilocalories (%)	52 (8.9)	52 (8.5)	53 (10)	50 (11)	.92
Protein intake (g)	73 (45)	73 (32)	82 (44)	96 (66)	.25
Fat intake (g)	91 (63)	84 (43)	89 (50)	95 (74)	.63
Saturated fat intake (g)	26 (18)	24 (13)	26 (15)	27 (18)	.77
Cholesterol intake (g)	0.30 (0.2)	0.27 (0.2)	0.30 (0.2)	0.37 (0.3)	.60
Fiber intake (g)	18 (11)	18 (8.2)	19 (10)	17 (13)	.68
Prevalent disease, year 1:					
Cardiovascular disease	13 (22%)	48 (35%)	26 (31%)	3 (21%)	.22
Hypertension	31 (56%)	82 (62%)	43 (54%)	10 (77%)	.60
Diabetes	13 (22%)	27 (20%)	17 (20%)	2 (14%)	.91
Cancer	4 (7%)	17 (12%)	11 (13%)	0	.46
Number of rx medications	3.4 (3.8)	3.3 (2.9)	3.1 (2.7)	3.0 (2.4)	.82
Taking lipid-lowering medication	5 (9%)	25 (18%)	14 (16%)	1 (7%)	.23
Overall lipid profile:					
Total cholesterol	183 (35)	198 (36)	200 (38)	193 (30)	.01; $\epsilon 2 < \epsilon 3, \epsilon 4$
Total cholesterol, year 1	175 (33)	199 (37)	199 (34)	186 (20)	<.0001; $\epsilon 2 < \epsilon 3, \epsilon 4$
LDL cholesterol, year 1	99 (33)	124 (34)	125 (33)	106 (18)	<.0001; $\epsilon 2 < \epsilon 3, \epsilon 4$

Table 1 (continued)

Mean (standard deviation) or frequency (percent)	<i>APOE</i> genotype categories				Overall <i>p</i> -value ^b ; pairwise comparisons ^c
	$\epsilon 2$ carriers (<i>n</i> =58)	$\epsilon 3$ homozygotes (<i>n</i> =137)	$\epsilon 4$ carriers (<i>n</i> =83)	$\epsilon 2\epsilon 4$ carriers (<i>n</i> =14)	
HDL cholesterol, year 1	53 (14)	52 (14)	51 (14)	59 (19)	.81
Triglycerides, year 1	120 (59)	116 (60)	116 (58)	103 (44)	.92
Interleukin-6 (pg/mL)	3.7 (4.5) Med=2.4	3.8 (3.6) Med=2.8	3.8 (3.6) Med=2.5	2.9 (1.8) Med=2.3	.69
Creatinine (mg/dL), year 1	1.3 (0.3)	1.2 (0.3)	1.3 (0.4)	1.2 (0.2)	.25

APOE, Apolipoprotein E genotype; Med, Median

^aMeasured at year 2 unless stated otherwise

^bExcluding *n*=14 participants with *APOE* genotype $\epsilon 2\epsilon 4$

^c $\epsilon 2$ denotes $\epsilon 2$ carriers ($\epsilon 2\epsilon 2$ and $\epsilon 2\epsilon 3$); $\epsilon 3$ denotes $\epsilon 3$ homozygotes ($\epsilon 3\epsilon 3$), and $\epsilon 4$ denotes $\epsilon 4$ carriers ($\epsilon 3\epsilon 4$ and $\epsilon 4\epsilon 4$)

creatinine, cystatin C, and triglycerides and plasma total and HDL cholesterol were measured at year 1 by the Laboratory for Clinical Biochemistry Research at the University of Vermont. LDL cholesterol was calculated using the Friedewald equation [28].

Statistical analysis

Differences in demographics, behavioral factors, and markers of disease by *APOE* were tested using analysis of variance for normally distributed continuous measures, Kruskal–Wallis test for non-normally distributed continuous measures, and chi-square test for categorical measures. We applied principal factor analyses with varimax rotation in SAS 9.4 separately by lipid categories (see Supplemental Table 3 for lipids organized by categories). For each lipid category, we calculated subject-specific weighted factor scores from the first factor. For lipid categories that did not contain enough metabolites for a factor analysis or if the first factor had an eigenvalue less than one, then we examined these metabolites individually in our main analyses (e.g., sphingosine, free carnitine, and C34:0 PS). In our previous work on metabolomics of walking ability in older adults [29], we found that the direction of associations between triacylglycerols and walking ability differed based on the degree of fatty acid saturation. Thus, to account for this, we calculated separate factor scores for triacylglycerols that did versus did not consist mostly of polyunsaturated fatty acids.

We identified metabolite factor scores and individual metabolites that differed by *APOE* using analysis of variance, adjusting for unequal variances when necessary ($p < 0.10$ from Levene's test for homogeneity). To account for multiple comparisons, we used a Benjamini–Hochberg correction [30] with 30% false discovery rate since this was a hypothesis-generating report [22, 31]. Pairwise comparisons by *APOE* were made when an overall difference in a metabolite was observed. As a sensitivity analysis, we examined differences in metabolite factor scores and individual metabolites by *APOE* when excluding participants taking lipid-lowering medication.

Results

Table 1 indicates minimal differences in study characteristics by *APOE*. Overall, 15% of the cohort was taking lipid-lowering medications, of which almost all of those participants were taking statins. Fewer $\epsilon 2$ carriers were taking lipid-lowering medications than $\epsilon 3$ homozygotes and $\epsilon 4$ carriers, though this difference was not statistically significant ($p = 0.23$). Total cholesterol was lower among $\epsilon 2$ carriers than $\epsilon 3$ homozygotes and $\epsilon 4$ carriers. HDL and LDL cholesterol and triglycerides were not measured at year 2 but were measured at year 1; $\epsilon 2$ carriers had lower LDL cholesterol, but there was no difference in HDL cholesterol nor triglycerides when compared to $\epsilon 4$ carriers or $\epsilon 3$ homozygotes.

Table 2 Associations between lipids and *APOE* genotype among Health ABC Black men

	Mean (standard error) of <i>APOE</i> genotype categories				Overall <i>p</i> -value ^a ; pairwise comparisons ^b
	$\epsilon 2$ carriers (<i>n</i> = 58)	$\epsilon 3$ homozygotes (<i>n</i> = 137)	$\epsilon 4$ carrier (<i>n</i> = 83)	$\epsilon 2\epsilon 4$ carriers (<i>n</i> = 14)	
Monoacylglycerol (MAG) score	−0.38 (0.10)	0.19 (0.10)	0.0007 (0.10)	−0.24 (0.16)	0.0005 ^c ; $\epsilon 2 < \epsilon 4$, $\epsilon 3$
Cholesteryl ester (CE) score	−0.38 (0.13)	0.08 (0.09)	0.14 (0.11)	−0.04 (0.26)	0.005 ^c ; $\epsilon 2 < \epsilon 3$, $\epsilon 4$
Sphingosine	−0.23 (0.13)	0.18 (0.08)	−0.06 (0.11)	−0.49 (0.26)	.02 ^c ; $\epsilon 2 < \epsilon 3$
Triacylglycerol (TAG) that consist mostly of polyunsaturated fatty acids score	0.29 (0.13)	−0.05 (0.09)	−0.15 (0.11)	0.17 (0.27)	0.03 ^c ; $\epsilon 2 > \epsilon 3$, $\epsilon 4$
Free carnitine	0.06 (0.13)	0.12 (0.09)	−0.23 (0.11)	−0.06 (0.27)	.04 ^c ; $\epsilon 4 < \epsilon 3$
PE plasmalogen score	0.23 (0.13)	−0.02 (0.09)	−0.17 (0.11)	0.25 (0.27)	0.07
Lysophosphatidylethanolamine (LPE) score	0.10 (0.12)	−0.15 (0.09)	0.10 (0.10)	0.46 (0.26)	0.11
PS plasmalogen score	−0.20 (0.13)	0.10 (0.09)	−0.04 (0.11)	0.11 (0.27)	0.16
Sphingomyelin (SM) score	−0.22 (0.13)	0.05 (0.09)	0.06 (0.11)	0.12 (0.27)	0.18
PC plasmalogen score	0.07 (0.13)	0.04 (0.09)	−0.18 (0.11)	0.40 (0.27)	0.22
3-methyladipate	0.21 (0.16)	−0.03 (0.08)	−0.10 (0.11)	−0.006 (0.25)	0.28
Phosphorylcholine (PC) score	−0.19 (0.13)	0.06 (0.09)	−0.008 (0.11)	0.24 (0.27)	0.30
Mevalonic acid	0.11 (0.13)	−0.09 (0.09)	0.07 (0.11)	−0.02 (0.27)	0.32
Lysophosphatidylcholine (LPC) score	0.05 (0.13)	−0.10 (0.09)	0.06 (0.11)	0.42 (0.27)	0.47
Alpha-glycerophosphocholine	0.13 (0.13)	−0.02 (0.09)	−0.08 (0.11)	0.12 (0.27)	0.48
Phosphatidylinositol (PI) score	−0.12 (0.13)	0.05 (0.09)	−0.03 (0.11)	0.15 (0.27)	0.57
Acylcarnitine score	0.04 (0.13)	0.05 (0.09)	−0.09 (0.11)	−0.11 (0.27)	0.61
Phosphatidylethanolamine (PE) score	0.09 (0.13)	−0.002 (0.09)	−0.04 (0.11)	−0.10 (0.27)	0.73
Bile acid score	−0.01 (0.13)	−0.0005 (0.09)	0.09 (0.11)	−0.49 (0.27)	0.75
Diacylglycerol (DAG) score	0.08 (0.13)	0.006 (0.08)	−0.04 (0.11)	−0.16 (0.27)	0.78
Alpha-glycerophosphate	0.01 (0.13)	−0.02 (0.09)	0.06 (0.11)	−0.18 (0.27)	0.86
C34:0 PS	−0.09 (0.13)	−0.002 (0.09)	−0.02 (0.11)	0.47 (0.27)	0.87
Adipate	0.02 (0.13)	−0.02 (0.09)	0.03 (0.11)	−0.11 (0.27)	0.94
Triacylglycerol (TAG) that do <i>not</i> consist mostly of polyunsaturated fatty acids score	0.02 (0.13)	0.006 (0.08)	−0.002 (0.11)	−0.14 (0.27)	0.99
Ceramide score	0.0007 (0.13)	−0.001 (0.09)	0.004 (0.11)	−0.01 (0.27)	0.999

APOE, Apolipoprotein E; *PE*, phosphatidylethanolamine; *PS*, phosphatidylserine; *PC*, phosphorylcholine

^aExcluding *n* = 14 participants with *APOE* genotype $\epsilon 2\epsilon 4$

^b $\epsilon 2$ denotes $\epsilon 2$ carriers ($\epsilon 2\epsilon 2$ and $\epsilon 2\epsilon 3$); $\epsilon 3$ denotes $\epsilon 3$ homozygotes ($\epsilon 3\epsilon 3$), and $\epsilon 4$ denotes $\epsilon 4$ carriers ($\epsilon 3\epsilon 4$ and $\epsilon 4\epsilon 4$)

^cFalse discovery rate < 0.30

Supplemental Table 4 includes the proportion of variance explained for each of the 17 metabolite factor scores. The two factor scores for triacylglycerols resulted in the highest proportion of variance explained, whereas the phosphatidylinositol factor score which consisted of five phosphatidylinositols had the lowest proportion of variance explained. Table 2 compares the 17 metabolite factor scores and the remaining log-transformed and standardized values for the eight individual metabolites by *APOE*. We found that the monoacylglycerol score, cholesteryl ester score, sphingosine, free carnitine, and the factor score for triacylglycerols that consist mostly of polyunsaturated fatty acids were significantly different by *APOE* ($p < 0.05$, false discovery rate < 0.30). When examining pairwise comparisons, we found $\epsilon 2$ carriers had lower average levels of the monoacylglycerol score, cholesteryl ester score, and sphingosine and higher average levels of the factor score for the triacylglycerols that consist mostly of polyunsaturated fatty acids when compared to remaining participants. Carnitine was significantly lower among $\epsilon 4$ carriers than $\epsilon 3$ homozygotes. We found consistent mean differences in the metabolite factor scores and individual metabolites by *APOE* when excluding participants taking lipid-lowering medication.

Supplemental Table 3 compares the average log-transformed and standardized values for all 222 individual metabolites by *APOE*. There were 23 metabolites that significantly differed by *APOE* ($p < 0.05$). Consistent with our main analysis using lipid-category factor scores, we found three monoacylglycerols, six cholesteryl esters, five triacylglycerols, sphingosine, and free carnitine differed significantly by *APOE*, in addition to four PE plasmalogens and one sphingomyelin (Supplemental Table 3). Among the triacylglycerols, we found only those consisting mostly of polyunsaturated fatty acids were significantly higher among $\epsilon 2$ carriers compared to $\epsilon 3$ homozygotes or $\epsilon 4$ carriers (Table 2 and Supplemental Table 3). To examine this pattern further, we plotted the mean difference among $\epsilon 2$ carriers versus non- $\epsilon 2$ carriers for all 55 triacylglycerols that were measured in the Health ABC study against the number of double bonds and the number of carbon atoms within each triacylglycerol. Figure 1C further illustrates that triacylglycerols that were higher among $\epsilon 2$ carriers tended to have five or more double bonds. We examined this same type of plot for

monoacylglycerols and cholesteryl esters. Figure 1A and B illustrate that regardless of the number of double bonds and carbon atoms, all cholesteryl esters and monoacylglycerols tended to be lower among $\epsilon 2$ carriers versus non- $\epsilon 2$ carriers.

Discussion

APOE $\epsilon 2$ carriers were lower on cholesteryl esters, monoacylglycerols, and sphingosine and were higher on triacylglycerols that consisted mostly of polyunsaturated fatty acids than remaining participants. Carnitine was the only metabolite that differed significantly between $\epsilon 4$ carriers and $\epsilon 3$ homozygotes, which was lower among $\epsilon 4$ carriers. We found consistent mean differences in the metabolite factor scores and individual metabolites by *APOE* when excluding the small percentage of participants taking lipid-lowering medications.

Differences in metabolites by *APOE* were previously examined among Australian older adults who were either $\epsilon 2$ carriers or $\epsilon 4$ carriers and were matched on age, gender, and lipid-lowering medication to those who were $\epsilon 3$ homozygotes [32]. Similarly, differences in metabolites by *APOE* were mainly due to differences in $\epsilon 2$ carriers. Specifically, the Australian $\epsilon 2$ carriers versus $\epsilon 3$ homozygotes were higher on two lysophosphatidylcholines and four phosphatidylethanolamines [32]. Lack of overlap in metabolites that differed by *APOE* in the previous versus current study are likely due to differences in lipid profiling platforms and could partly be due to differences in demographics; our cohort consisted of U.S. Black men, whereas the previous report was an Australian population-based cohort of men and women, where Australia has a very small percentage of individuals of African ancestry [33]. There was also a higher proportion of participants taking lipid-lowering medication in the previous report than among the Health ABC Black men (55% versus 15%, respectively), and the previous report found no differences in total and LDL cholesterol by *APOE*. Future studies in older adult cohorts that use similar lipid profiling methods will be needed to replicate results.

Higher triglycerides have been reported among $\epsilon 2$ carriers versus $\epsilon 3$ homozygotes [6]. Using lipid profiling, we sought to identify triacylglycerols that

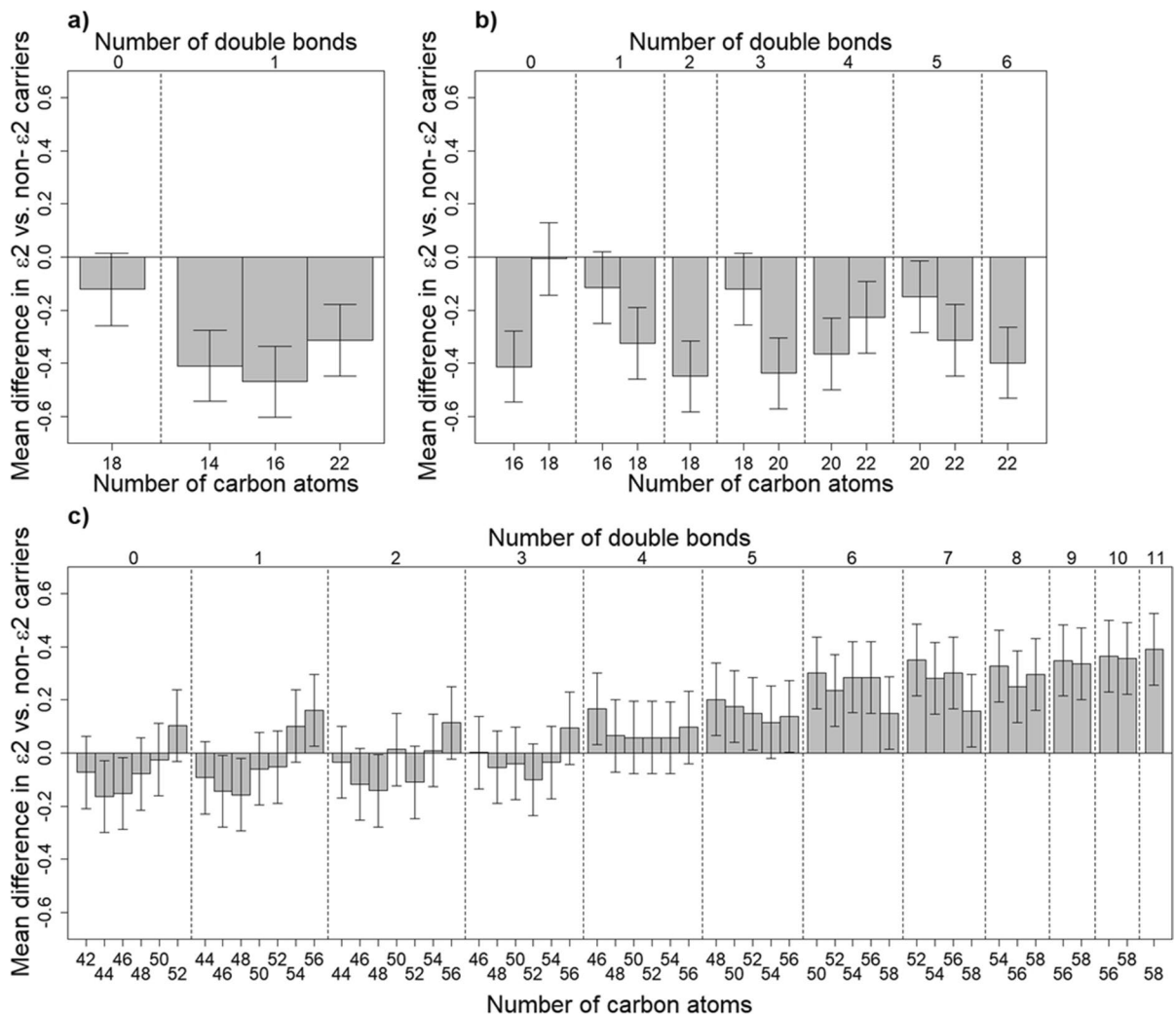


Fig. 1 Standardized mean (\pm standard error) difference in apolipoprotein E (APOE) ϵ 2 vs. non- ϵ 2 carriers for all available **A** monoacylglycerols, **B** cholesteryl esters, and **C** triacyl-

glycerols, sorted by number of double bonds and carbon atoms among Health ABC Black men

potentially contribute to this known difference in overall triglycerides by APOE. Triacylglycerols contain three fatty acids, and in this report, triacylglycerols that consist mostly of polyunsaturated fatty acids were higher among ϵ 2 carriers than ϵ 3 homozygotes and ϵ 4 carriers. A previous report examining fatty acid remodeling in cell membranes in response to different APOE isoforms [34] found that exposing human neuroblastoma cells to the APOE ϵ 3 or ϵ 4 isoforms resulted in decreased total polyunsaturated fatty acids and increased total saturated and monounsaturated fatty acids [34]. In addition, we previously

found triacylglycerols consisting mostly of polyunsaturated fatty acids were higher among older adults with high versus low walking ability [29], and others have noted these metabolites associated with less insulin resistance [35], lower waist circumference [35], and lower type 2 diabetes risk [36]. Higher triacylglycerols consisting mostly of polyunsaturated fatty acids may indicate more optimal fats available to ϵ 2 carriers and may be one mechanism contributing to lower morbidity and mortality among ϵ 2 carriers, despite having higher overall triglycerides than ϵ 3 homozygotes [11].

Triacylglycerols can be resynthesized or modified by the monoacylglycerol pathway [37]. Monoacylglycerols are classified based on fatty acid location; if the fatty acid is located on either end (i.e., 1- or 3-isomer), then it is an α -monoacylglycerol, whereas if the fatty acid is centered (i.e., 2-isomer), then it is a β -monoacylglycerol. The preferred substrates for the monoacylglycerol pathway are β -monoacylglycerols, which are the major end-product of dietary fat digestion in the small intestine and are directly converted to triacylglycerols by the monoacylglycerol pathway [37]. Monoacylglycerols are present in low levels and typically do not accumulate due to strong detergent properties [38]. Here, *APOE* $\epsilon 2$ carriers had lower levels of three of the four measured monoacylglycerols, whereas dietary fat intake did not differ. All monoacylglycerols measured in this report were α -monoacylglycerols, which can be formed by β -monoacylglycerols undergoing a random isomerize [39]. Though little else is known about α -monoacylglycerol, including what lower plasma α -monoacylglycerol among *APOE* $\epsilon 2$ carriers may indicate and how lower levels relate to β -monoacylglycerols and triacylglycerols.

In addition to higher overall triglycerides, lower levels of total and LDL cholesterol have been reported among *APOE* $\epsilon 2$ carriers versus $\epsilon 3$ homozygotes [6]. Here, a cholesteryl ester factor score was significantly lower among $\epsilon 2$ carriers than $\epsilon 4$ carriers or $\epsilon 3$ homozygotes. Excess cholesterol is stored primarily as cholesteryl esters [40], which make up a major component of macrophage foam cells of atherosclerotic plaques [41]. For example, one study found total cholesteryl esters were 120 times higher in atherosclerotic plaques than in healthy radial arteries [42], with the majority of cholesteryl esters in plaques being C18:1 and C18:2 [42]. Here, both plasma C18:1 and C18:2 cholesteryl esters were significantly lower among $\epsilon 2$ carriers than $\epsilon 4$ carriers or $\epsilon 3$ homozygotes. Some reports have also found a protective association between the *APOE* $\epsilon 2$ allele and cardiovascular outcomes. For example, a meta-analysis of 121 studies with coronary outcomes and over 120,000 participants reported $\epsilon 2$ carriers had 20% lower odds of coronary disease than $\epsilon 3$ homozygotes [10]. Similarly, *APOE* $\epsilon 2$ carriers had a 35% lower risk of mortality due to cardiovascular disease than $\epsilon 3$ homozygotes among a large older adult cohort with a good representation of the $\epsilon 2$ allele and more than 18 years of follow-up [15]. Large prospective

older adult cohorts with metabolomics and power to examine differences in incident cardiovascular disease risk by *APOE* will be able to further examine the protective effect of lower cholesteryl esters on cardiovascular outcomes among *APOE* $\epsilon 2$ carriers.

We found *APOE* $\epsilon 4$ carriers had lower levels of carnitine than $\epsilon 3$ homozygotes. Carnitine is a critical molecule in the mitochondrial metabolism of fatty acids. The most important function of carnitine is to transport long-chain fatty acids into the mitochondria [43] to be oxidized and used as an energy source for myocardium and skeletal muscle [38]. Carnitine can be synthesized in the body, but the majority comes from dietary animal-based sources, primarily from red meat and dairy products [38]. In a healthy individual, plasma carnitine and its acylcarnitine derivatives are tightly regulated within a fixed range, where altered levels, specifically lower carnitine, can be indicative of mitochondrial dysfunction [38, 43]. As a result, carnitine or acetylcarnitine supplementation has been suggested as a potential therapy to combat aging-related decline in mitochondria [44]. Mitochondrial dysfunction is thought to be involved in the pathophysiology of several aging-related major health outcomes, including Alzheimer's disease [45, 46] and cardiovascular disease [47]. Thus, mitochondrial dysfunction may partially explain the higher risk of morbidity and mortality associated with the *APOE* $\epsilon 4$ allele and might be mitigated through carnitine supplementation. However, a potential undesirable effect of carnitine supplementation is an increase in trimethylamine oxide (TMAO) [48], a gut microbiota-dependent metabolite of carnitine, which is associated with cardiovascular disease [49]. In fact, supplementation with TMAO or TMAO-related metabolites (e.g., carnitine) promoted atherosclerosis in animal models [48, 49]. It remains to be determined whether a lower dose of carnitine can improve mitochondrial functioning, while reducing the risk of accelerated atherosclerosis formation.

Only one metabolite differed significantly between *APOE* $\epsilon 4$ carriers and $\epsilon 3$ homozygotes in this cohort of older Black men. This is likely due to our relatively small number of $\epsilon 4$ carriers, limiting power to detect smaller effect sizes. On the other hand, we identified several differences in metabolites between $\epsilon 2$ carriers versus remaining participants, despite an even smaller number of $\epsilon 2$ carriers than the number of $\epsilon 4$ carriers.

This discrepancy is likely explained by the $\epsilon 2$ allele causing a 50 times lower affinity for apolipoprotein E to bind to LDL particles when compared to the $\epsilon 3$ and $\epsilon 4$ alleles [3]. This dramatic difference in lipoprotein affinity could result in much larger differences in circulating lipids among $\epsilon 2$ carriers, i.e., larger effect sizes that are easier to detect with smaller sample sizes. Multiple metabolites likely differ between *APOE* $\epsilon 4$ carriers and $\epsilon 3$ homozygotes that will need to be explored in a larger cohort.

In addition to the small number of $\epsilon 4$ carriers, we were limited by only having information on older Black men who were recruited to be non-disabled at baseline, it is unknown whether the identified metabolites that differed by *APOE* would generalize to all older adults, in particular to women, other racial/ethnic groups, or similarly aged older adults of poorer health than those meeting the eligibility criteria for this study. In addition, metabolite values were unitless peak areas, which limited the interpretability of the observed differences by *APOE*; for example, we were not able to assess whether the lower level of carnitine among $\epsilon 4$ carriers versus $\epsilon 3$ homozygotes was outside of a healthy range. Strengths of this work included the higher proportion of $\epsilon 2$ carriers when compared to U.S. frequencies, as well as a well-characterized cohort of older Black men with a large number of metabolites available from overnight-fasting plasma samples that had never been thawed previously.

The observed differences in lipids provide a better characterization of metabolic differences according to *APOE* in this cohort of older Black men. Lower levels of cholesteryl esters, monoacylglycerols, and sphingosine and higher levels of triacylglycerols that consist mostly of polyunsaturated fatty acids may be a protective profile of lipids among *APOE* $\epsilon 2$ carriers. Specifically, the higher triacylglycerols consisting mostly of polyunsaturated fatty acids among *APOE* $\epsilon 2$ carriers may indicate more optimal fats available in the periphery to be transported to tissues as an energy source and the lower cholesteryl esters potentially indicate less cholesterol available in the periphery to accumulate as atherosclerotic plaques. It remains to be determined whether this potentially protective profile of lipids contributes to the *APOE* $\epsilon 2$ allele-associated lower risks of Alzheimer's disease, cardiovascular disease, and mortality. In addition, the observed lower circulating carnitine among *APOE* $\epsilon 4$ carriers versus $\epsilon 3$ homozygotes may potentially reflect lower mitochondrial functioning

among $\epsilon 4$ carriers that might explain a portion of the higher risk of morbidity and mortality associated with the $\epsilon 4$ allele. A complete understanding of differences in lipid metabolism that occur as a result of *APOE* allelic variation could potentially indicate metabolic pathways involved in pathophysiology of multiple major health outcomes.

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Data availability Data and material are available by submitting an analysis proposal at <https://healthabc.nia.nih.gov/>.

Code availability Not applicable.

Declarations

Ethics approval The Health ABC study was approved by the Institutional Review Board of each participating site.

Consent to participate All Health ABC participants provided written informed consent.

Consent for publication All authors and the Health ABC study approved this manuscript for publication.

Conflict of interest The authors declare no competing interests.

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