Clinical and Epidemiologic Research

Acylcarnitine Abnormalities Implicate Mitochondrial Dysfunction in Patients With Neovascular Age-Related Macular Degeneration

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Purpose. Abnormalities in lipid metabolism are implicated in age-related macular degeneration (AMD), but the pathways involved remain unclear. We assessed whether acylcarnitine concentrations, a marker of lipid and mitochondrial metabolism, differed between patients with AMD and controls.

Methods. In this cross-sectional case-control study, cases (n = 81) had neovascular AMD and controls (n = 79) had cataract with no other ocular pathology. Participants were recruited from eye clinics in Western Sydney, Australia, between 2016 and 2018. Plasma blood samples were collected and liquid chromatography mass spectrometry analyses performed to identify acylcarnitine concentrations. Acylcarnitine levels were adjusted for age, gender and smoking in multivariable models. Confirmation of key acylcarnitine identities was conducted using high mass accuracy liquid chromatography-tandem mass spectrometry.

Results. After multivariable adjustment, C2-carnitine (acetylcarnitine) levels were significantly lower in patients with neovascular AMD compared to controls (0.810 ± 0.053 (standard error) compared to 1.060 ± 0.053), p = 0.002). C18:2-DC carnitine (a dicarboxylic acylcarnitine with a 18 carbon side chain and 2 double bonds), levels were significantly higher in patients with neovascular AMD compared to controls (1.244 ± 0.046 compared to 1.013 ± 0.046), p = 0.001). Other acylcarnitines examined were not significantly different between cases and controls.

Conclusions. Reduced plasma levels of C2-carnitine (acetylcarnitine) and increased plasma levels of C18:2-DC carnitine were observed in patients with neovascular AMD compared to controls. These findings suggest mitochondrial dysfunction could be involved in the pathogenesis of neovascular AMD.

Keywords: macular degeneration, acylcarnitines, mitochondria

Age-related macular degeneration (AMD) remains a major cause of blindness worldwide. A number of genetic risk factors for AMD have been discovered, as well as environmental risk factors such as smoking and low dietary intake of certain lipids, antioxidants, lutein, and zeaxanthin.[1,2] What remains unclear is how these risk factors interact to cause the disease. The pathogenic pathways that cause AMD remain incompletely understood, and a deeper understanding of these pathways is needed.

A number of studies have shown that a diet rich in oily fish reduces the risk of developing late AMD.[2–7] Other studies have demonstrated that lipid metabolites influence angiogenesis in neovascular eye diseases such as AMD.[8] In particular, ω-3 and ω-6 long-chain polyunsaturated fatty acids and their metabolites may play important roles in regulating angiogenesis.[9,10] Acylcarnitines are markers of lipid metabolism in the mitochondria and may also be dysregulated in AMD pathogenesis.

Acylcarnitines are the catabolic end products of fatty acids and branched-chain amino acids.[11,12] They are derived from exchange of acyl groups of different lengths between coenzyme A (co-A) and L-carnitine catalyzed by a series of carnitine acyl-transferases in mitochondria. These transferases have overlapping chain length specificity for acyl groups, ranging from 2 carbons (acetylcarnitine or C2 carnitine) to over 18 carbons (stearoylcarnitine or C18 carnitine) in length. Acylcarnitines function to transport acyl groups from fatty acids and branched-chain amino acids into mitochondria to generate cellular energy. Unlike their corresponding acyl-CoA analogues, acylcarnitines are able

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to cross mitochondrial and cell membranes via the carnitine shuttle[12] and are readily detectable in plasma, where their levels approximate that in the mitochondrial matrix.[13]

A severe block in the catabolic pathways of either the fatty acids or branched-chain amino acids can result in accumulation of one or more acylcarnitines in blood plasma. Analysis of plasma acylcarnitines can detect more than 25 metabolic disorders of fatty acid and branched-chain catabolism.[14,15] Examples of disorders related to defects in acylcarnitine metabolism include short-, medium-, long-, and very long-chain acyl-Co-A dehydrogenase deficiencies. These syndromes are highly variable and often present with encephalopathy, cardiomyopathy, and myopathy, disorders of highly metabolically active organs. More recently, milder versions of acylcarnitine abnormalities have been detected in chronic diseases such as insulin resistance, cardiovascular disease,[16,17] and Alzheimer disease,[18] where they may play a pathogenic role.[19]

As the retina is the most metabolically active tissue in the body with a high concentration of mitochondria and lipids,[20] we hypothesized that a defect in acylcarnitine metabolism may result in pathology such as AMD. We therefore conducted a study to determine whether acylcarnitine concentrations in plasma are altered in patients with AMD.

Methods

Patient Population and Sample Collection

The study design is a case-control study of patients with neovascular AMD compared to age- and gender-matched controls without any AMD. Cases were recruited from two neovascular AMD treatment clinics in Western Sydney while controls were recruited from the cataract clinics of Westmead Hospital, Sydney, Australia. Patients were recruited from May 2016 to May 2018. Cases had to meet inclusion criteria, including consultant-confirmed diagnosis of neovascular AMD in at least one eye, imaging (fundus fluorescein angiography or spectral domain optical coherence tomography confirming the presence of neovascular AMD), no geographic atrophy in either eye, and ability to consent to the study. Controls had no ocular pathology other than cataract and in particular had to have no signs of early or late AMD. Exclusion criteria for both cases and controls include presence of eye disease that could confound results such as diabetic retinopathy, retinal vein occlusion, epiretinal membrane, previous retinal detachment, or previous ocular neoplasm. Further exclusion criteria included presence of systemic comorbidities that could confound lipidomic analyses such as diabetes, any cancer except fully excised skin cancer, kidney disease, and liver disease. All participants provided signed informed consent, and the study was approved by the Human Research Ethics Committees of Western Sydney Local Health District and the University of Sydney.

Participants completed an interviewer-administered medical questionnaire that collected demographic and medical history information. Further medical and ocular history was obtained from hospital and clinic records. Time of the last three meals and meal contents were recorded to determine fasting status. A fasted state was defined as a last meal 6 hours or more prior to blood collection. Venous blood samples were taken from the antecubital vein and immediately stored on ice or in a 4°C (Celsius) fridge. Blood samples were then centrifuged at 1500 × g for 20 minutes at 4°C to obtain plasma, which was then stored in a −80°C freezer within 6 hours of blood collection. Samples were defrosted and analyzed in batches to ensure uniform processing and lipid extraction.

Lipid Extraction and Acylcarnitine Derivatization

Acylcarnitine analysis of patient plasma was conducted following a previously established protocol.[21] In brief, 100 µL of patient plasma was spiked with the internal standard, d3-palmitoylcarnitine, with a final concentration of 200 nM (Cambridge Isotope Laboratories, Tewksbury, Massachusetts, USA). Then, 800 µL of methanol was added to samples, vortexed, and centrifuged to promote protein precipitation. Next, 100 µL of 3 M methanolic HCl (Sigma, St. Louis, MO, USA) was added to 200 µL of the acylcarnitine-containing supernatant for methyl ester derivatization. The samples were dried and reconstituted in 100 µL of 85% methanol.

Flow-Injection Tandem Mass Spectrometry

Samples were analyzed by a modified version of the flow analysis described by Millington and Stevens.[21] A Sciex QTRAP 6500 (Waverley, Victoria, Australia) tandem mass spectrometer coupled to a Shimadzu LC-30AD (Sydney, New South Wales, Australia) was used to flow-inject samples using a mobile phase of 50:50 methanol and water at 20 µL/min for 7 minutes. The mass spectrometer used a declustering potential of 150 V, ion spray voltage of 5500 V, and collision energy of 46 to conduct a precursor ion scan of *m/z* 99.1. The scan was conducted with the turbo spray source at 21°C and recorded as an accumulated spectrum from *m/z* 200 to 500. Gas settings were set at curtain gas (CUR) of 20, GS1 of 15, and GS2 of 15.

Areas under the peak for each acylcarnitine were calculated using a summation integration method in MultiQuant 3.0 (Waverley, Victoria, Australia). The areas for each acylcarnitine were analyzed as ratios to the internal standard, d3-palmitoylcarnitine, to generate relative concentrations. To account for interexperimental variability, the mean of the control samples was calculated and used to divide the relative concentration of acylcarnitine for each sample within the same experiment. This normalization was conducted for each experiment prior to pooling the data.

Corroborative High-Resolution Liquid Chromatography Tandem Mass Spectrometry

To confirm the assigned identity of select acylcarnitine species, a second method, incorporating liquid chromatography and accurate mass measurements with parallel reaction monitoring, was employed. A Thermo Fisher Scientific Orbitrap HFX mass spectrometer was interfaced to a Thermo Fisher Scientific Vanquish liquid chromatography (LC) system. Then, 5 µL of derivatized sample was injected on a 2.1 × 100-mm, C18 LC column (Thermo Fisher Scientific, Scoresby, Victoria, Australia) with solvent delivery at 100 µL/min. A gradient of 20% solvent A (0.1% formic acid in water) to 100% solvent B (0.1% formic acid in acetonitrile) was run over 10 minutes. High-resolution (60,000) mass spectra were collected over a 150- to 500-m/z range. A list of 17 masses covering the

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Table 1. Baseline Characteristics of Patients in the Study

Characteristic	Cases With Neovascular Macular Degeneration	Controls	*P* Value
Number	81	79	
Mean (SD) age, y	78.7 (8.6)	73.9 (10.7)	<0.0001
Male, %	45.7	53.4	0.33
Current smoking, %	8.0	18.8*	0.001
Confirmed fasting >6 hours, %	50	53	0.67
Hypertension, %	54.2	65.3	16.1
Previous coronary heart disease, %	16.9	16.8	0.97
Previous stroke, %	9.3	13.9	0.38
Bilateral neovascular macular degeneration, %	42	0	<0.0001

* More current smokers in controls likely because cases were told to stop smoking.

target acylcarnitines were monitored and product ion scan collected over 50 to 600 m/z at 15,000 resolution.

Statistical Methods

Normalized concentrations of acylcarnitines were compared between cases and controls using the nonparametric Kruskal-Wallis test. Multivariate analyses adjusting for age, gender, and smoking were performed as these confounders are associated with AMD. Adjusted means and standard errors are reported. Results are reported with a two-tailed significance level threshold of $P = 0.05$. SAS (version 9.1; SAS Institute, Cary, NC, USA) was used for all analyses.

Results

Patient Population

The study recruited 81 cases and 79 controls who provided usable data on AMD status and lipidomic experiments. The mean (SD) age was 78.7 (8.6) years for cases and 73.9 (10.7) years for controls (Table 1). Cases were less likely to be current smokers, likely because cases had previously been advised to stop smoking; 42% of cases had bilateral neovascular Age-related macular degeneration (nAMD).

Acylcarnitine Analyses

Figure shows the acylcarnitine analysis by flow-injection mass spectrometry. Figure A is a representative spectrum obtained from acylcarnitine precursor ion scan with magnified views in the insets. Several of the major carnitine peaks have been annotated, including C2, C18:1, and C18:2-DC carnitine. The representative extracted ion chromatographs for C2 carnitine and C18:2-DC carnitine from both control (black line) and AMD (red line) are shown in greater detail in Figure B. In Figure C, individual patient areas under the peak for C2 carnitine and C18:2-DC carnitine are plotted as a fold change of control patient samples. C2 carnitine concentrations are lower, while C18:2-DC carnitines concentrations are higher in patients with AMD compared to controls. There was some overlap between the values, but using the nonparametric Kruskal-Wallis test, C2 carnitine levels were significantly lower ($P = 0.04$) while C18:2-DC levels were strongly significantly higher ($P = 0.0008$) in AMD cases compared to controls. We divided the population into three age groups (<60 years, 80 years, and >80 years) and did not detect a difference in acylcarnitine concentrations between the age groups.

Analyses Adjusted for Age, Gender, and Smoking

Table 2 shows the results after adjusting for age, gender, and smoking status. The associations for C2 carnitine and C18:2-DC carnitine were replicated and strengthened. C2 carnitine levels were significantly lower in patients with neovascular AMD compared to controls (0.810 ± 0.053 [standard error] compared to 1.060 ± 0.053, $P = 0.002$). C18:2-DC levels were significantly higher in patients with neovascular AMD compared to controls (1.244 ± 0.046 compared to 1.013 ± 0.046, $P = 0.001$). A modest association was also observed for C18:1-DC, which was higher in patients with neovascular AMD (1.237 ± 0.061 compared to 1.053 ± 0.061, $P = 0.044$). Other acylcarnitines examined were not significantly different between cases and controls (Table 2).

Analyses Excluding Nonfasted Patients

We performed further supplementary analyses by excluding patients who were not fasted (last meal less than 6 hours prior). These analyses used data from 39 cases and 39 controls. Both raw and multivariable analyses adjusting for age, gender, and smoking confirmed the findings that C2 carnitine and C18:2-DC carnitine were significantly different between cases and controls, and results were similar to those in Table 2 (multivariable adjusted means for C2 carnitine in cases [0.858 ± 0.078] and controls [1.136 ± 0.0762], $P = 0.01$; for C18:2-DC carnitine cases [1.248 ± 0.067] and controls [1.001 ± 0.065], $P = 0.02$). The main difference was a reduction in the significance level for both acylcarnitines to $P = 0.02$ for both.

Corroborative High Mass Accuracy LC Mass Spectrometry. Due to the low resolution of the triple quadrupole mass spectrometer employed, the flow-injection mass spectrometry (MS) method cannot differentiate between isobaric acylcarnitine species. For example, the nominal *m/z* (as its methyl ester) of C18:2-DC carnitine is 482, the same as another carnitine species, C20:2-OH carnitine. To distinguish between these two possibilities, an additional high-resolution high mass accuracy LC-MS method was used to confirm the identities of all assignments in Table 2.

The mass accuracy obtained by the instrument used in this method is sufficient to distinguish between C18:2-DC carnitine (calculated mass 482.3481) and C20:2-OH carnitine (calculated mass 482.3845). The observed mass from a representative case sample was 482.3491, within 1.3 ppm of the expected C18:2-DC mass. That this 482 species is an acylcarnitine was further confirmed by

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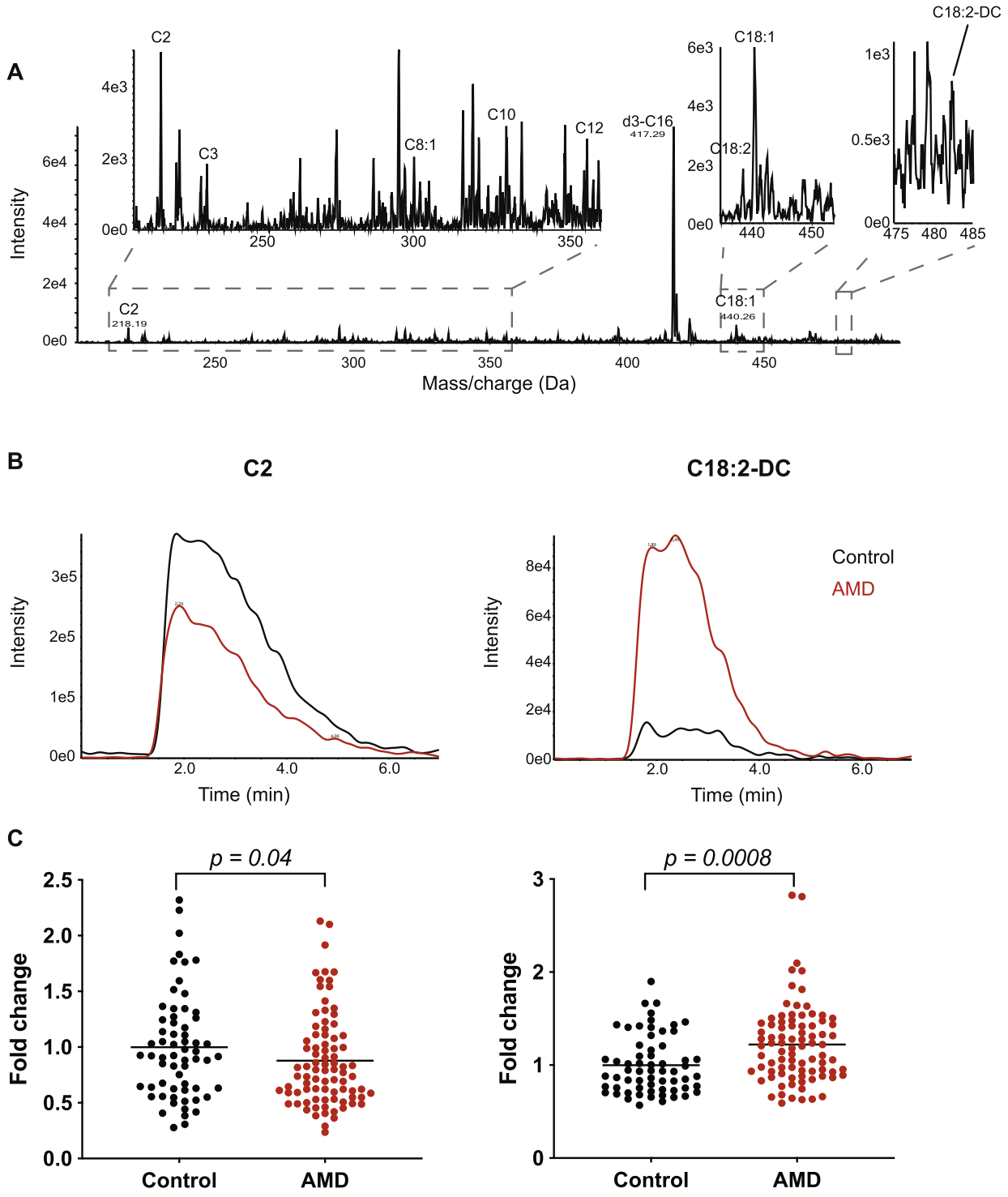

Figure. Acylcarnitine analysis by flow-injection mass spectrometry. (**A**) Representative spectrum obtained from acylcarnitine precursor ion scan. (**B**) Representative summation peaks for C2 and C18:2-DC. (**C**) Individual patient area under the peak for C2 and C18:2-DC plotted as a fold change of control patient samples. Raw values without adjusting for age, gender, and smoking were used. Statistics conducted using a Kruskal-Wallis test.

observing the expected signature fragment ion at 99.0445 within 1 ppm. The generic name C18:2-DC carnitine is used as the double-bond locations or stereochemistry in the C18 side chain could not be determined in this analysis.

Discussion

Acylcarnitines are essential for energy production by mitochondria, and the retina is the most metabolically active tissue in the body. This study found that a short-chain acetyl-

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Table 2. Acylcarnitine Levels Normalized to Controls and Adjusted for Age, Gender, and Smoking

Acylcarnitine Levels (Adjusted for Age, Gender, and Smoking)	Controls, Mean (SE)	Cases With Neovascular Macular Degeneration, Mean (SE)	*P* Value
C:2	1.060 (0.053)	0.810 (0.053)	0.002
C:3	1.025 (0.065)	0.857 (0.065)	0.08
C:4	1.126 (0.086)	1.014 (0.0835)	0.38
C:5	1.004 (0.073)	1.027 (0.073)	0.86
C:6	1.049 (0.063)	0.889 (0.063)	0.09
C:8	1.059 (0.098)	0.954 (0.098)	0.48
C:10	1.078 (0.101)	0.986 (0.101)	0.54
C:12	1.072 (0.066)	0.920 (0.066)	0.13
C:14	1.038 (0.048)	0.954 (0.048)	0.25
C:16	1.006 (0.036)	0.953 (0.036)	0.34
C:16_2	1.029 (0.049)	0.964 (0.049)	0.38
C:18	1.014 (0.043)	1.053 (0.043)	0.54
C:18_1_DC	1.053 (0.061)	1.237 (0.061)	0.044
C:18_2_DC	1.013 (0.046)	1.244 (0.046)	0.001
C:20	0.993 (0.050)	1.098 (0.050)	0.16
C:22	0.973 (0.037)	1.061 (0.037)	0.12

The abundances of C3, C4, and C6 were very low, and the results for these acylcarnitines may not be reliable.

carnitine (C2) was deficient by 24% in patients with neovascular AMD compared to controls without AMD. A long-chain acylcarnitine (C18:2-DC) was elevated by 23% in patients with neovascular AMD. These associations persisted when analyses were confined to patients who had fasted for at least 6 hours prior, reducing the possibility of confounding by recent meals.

C2 carnitine or acetylcarnitine is the main carnitine found in plasma and is the major substrate for mitochondrial oxidative phosphorylation.[22] The deficit of acetylcarnitine suggests impaired mitochondrial function, as acetylcarnitine is produced in the mitochondrial intermembrane space. Reduction in acetylcarnitine has been linked to increased rates of neurodegenerative disease such as Alzheimer dementia,[18] peripheral neuropathy,[23] and major depressive disorder.[24] To our knowledge, this is the first report linking a deficiency to neovascular AMD, and it supports a pathogenic role for mitochondrial dysfunction in neovascular AMD.

Mitochondrial dysfunction in AMD may be related to increased oxidative stress from environmental exposures such as smoking and chronic low-grade inflammation at Bruch's membrane from the upregulated alternative complement pathway in patients with the high-risk complement factor H (CFH) allele.[25] Compared to nuclear DNA, mitochondrial DNA is more susceptible to mutations from increased oxidative stress because it lacks protective histones and has fewer DNA repair mechanisms. Impaired mitochondrial DNA can result in abnormal mitochondrial function and morphology, both of which have been observed in patients with AMD.[26,27] Some studies suggest mitochondrial dysfunction may play a role in triggering choroidal neovascularization in AMD.[27–30] Other studies have suggested that the gene product for a major AMD susceptibility gene, ARMS2, is located in the mitochondria.[31,32] Finally, mutations in mitochondrial genes themselves have been linked to increased risk of AMD, potentially through impaired mitochondrial energy production or through "back signaling" with altered nuclear expression of high-risk AMD genes.[29,33–36]

There is evidence that age-related, multifactorial, neurodegenerative diseases such as AMD and Alzheimer dementia are linked through common mitochondrial dysfunction. Epidemiological studies have consistently shown an association between AMD and Alzheimer dementia,[37–39] and increasing evidence implicates mitochondrial dysfunction as contributing to the risk of both diseases.[40,41]

This study also found elevated levels of C18:2-DC carnitine in patients with neovascular AMD. This molecule is composed of a dicarboxylic carnitine with an 18-carbon acyl group with two double bonds. The significance of elevated levels in patients with neovascular AMD is unclear. Linoleic acid (C18:2) is a possible precursor of C18:2-DC and is an essential ω-6 fatty acid found in many vegetable oils such as sunflower, corn, and soybean oils, which have been linked with greater risk of late AMD in some studies,[3–5] although not confirmed in other studies.[6] Our finding of higher C18:2-DC in patients with neovascular AMD may help explain this finding, as well as suggesting that long-chain ω-6 fatty acid pathways involving linoleic acid derivates may play a role in the pathophysiology of neovascular AMD.

A recently published study found carnitine levels were 28% lower in aqueous humor of patients with neovascular AMD compared to controls,[42] providing further support for our findings implicating carnitine metabolism and mitochondrial dysfunction in neovascular AMD. Patients with Alzheimer dementia and cognitive impairment have serum acetylcarnitine (C2 carnitine) levels 36% and 27% lower than healthy controls, respectively, while C18:2 levels were 21% and 14% lower than in healthy controls, respectively.[18] Another study found differences of between 18% and 39% in different acylcarnitine levels in patients with Alzheimer dementia compared to controls.[43] Patients with major depressive disorder have 20% lower levels of acetylcarnitine (C2) in their plasma than healthy controls.[24]

Differences in metabolites of this magnitude are expected in multifactorial diseases, where they may be biomarkers of reduced mitochondrial function. This reduction in mitochondrial efficiency, acting in concert with other risk factors (e.g., smoking, proinflammatory milieu, genetic predisposition), may then trigger the onset of multifactorial disease such as neovascular AMD. We did not expect large reductions in acylcarnitine levels as this would lead to life-threatening pathology such as hypoglycemia, skeletal and cardiomyopathy, and hepatic encephalopathy, as occur in

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patients with systemic primary carnitine deficiency.[44] There is some overlap between the levels of acylcarnitines between nAMD cases and controls. This is common in multifactorial diseases as small changes in a number of molecules may act synergistically or additively to cause the disease phenotype.[45]

Our results have some clinical implications. Acetylcarnitine (C2 carnitine) is a small, water-soluble molecule able to cross the blood-retinal barrier freely. Oral supplementation could help increase mitochondrial concentrations and improve retinal energy metabolism.[46] A small randomized clinical trial found oral supplementation with a combination of acetylcarnitine, n-3 fatty acids, and coenzyme Q10 improved visual function in patients with early AMD with some regression of drusen, although it was unclear whether this was due to acetylcarnitine or the other agents.[47] Acetylcarnitine also functions as a carbon donor, providing methyl groups for histone methylation and influencing gene expression. In the brain, this stimulates membrane phospholipid synthesis, exerts antiapoptotic activity, modulates production of neurotrophic factors, and protects neurons from excitotoxicity.[22,23] Reduced levels could potentially predispose to AMD and may contribute to retinal pigment epithelium and photoreceptor degeneration underlining the disease through these above mechanisms. If replicated in other studies, acetylcarnitine (C2 carnitine) and C18:2-DC carnitine may potentially be useful as blood biomarkers of patients at risk of developing neovascular AMD.

Strengths of this study include its moderately large sample size, careful selection of cases and controls with confirmation of fasting status, adjustment for confounders, and uniform and masked processing of both cases and controls. Some limitations should be considered. First, the study design is a case control, so cause and effect cannot be definitely established. Nonetheless, it is unlikely that neovascular AMD causes lower levels of acetylcarnitine (C2 carnitine) and higher levels of other longer chain acylcarnitines. Second, there may be unmeasured confounders that influenced the results. We excluded as many of these as we could by excluding patients with kidney disease, cancer, and diabetes. Third, we did not examine patients with atrophic (dry) AMD, so it is unclear if these findings would also apply to patients with dry AMD. Future prospective studies will help address these limitations and confirm these findings. Finally, we use the generic name C18:2-DC carnitine as the double-bond locations or stereochemistry in the C18 side chain could not be determined in this analysis.

In summary, we report reduced levels of acetylcarnitine (C2 carnitine) and increased levels of C18:2-DC carnitine in patients with neovascular AMD compared to controls without AMD. These results suggest mitochondrial dysfunction may be involved in the pathogenesis of neovascular AMD.

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