Blood Biomarkers as Surrogate Endpoints of Treatment Responses to Aerobic Exercise and Cognitive Training (ACT) in Amnestic Mild Cognitive Impairment: The Blood Biomarkers Study Protocol of a Randomized Controlled Trial (The ACT Trial)

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Danni Li
dannili@umn.edu Corresponding Author

Michelle M Mielke
Mayo Clinic School of Medicine

W Robert Bell
University of Minnesota

Cavan Reilly
University of Minnesota

Lin Zhang
University of Minnesota

Feng Yankee Lin
University of Minnesota

Fang Yu
University of Minnesota

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Abstract

Background: Alzheimer’s disease (AD) is an epidemic with tremendous public health impacts because there are currently no disease-modifying therapeutics. Randomized controlled trials (RCTs) for prevention of AD dementia often use clinical endpoints that take years to manifest (e.g., cognition) or surrogate endpoints that are costly or invasive (e.g., magnetic resonance imaging MRI). Blood biomarkers represent a clinically applicable alternative surrogate endpoint for RCTs that would be both cost-effective and minimally invasive, but little is known about their value as surrogate endpoints for treatment responses in the prevention of AD dementia.

Methods: The objective of this study is to investigate blood neuropathological, neurodegenerative, and neurotrophic biomarkers as surrogate endpoints for treatment responses to 3 interventions in older adults with amnestic mild cognitive impairment (aMCI, a prodromal stage of AD): aerobic exercise; cognitive training; and combined aerobic exercise and cognitive training (ACT). We chose these three sets of biomarkers for their unique mechanistic associations with AD pathology, neurodegeneration and neurogenesis. This study is built on the ACT Trial (1R01AG055469), a single-blinded, multi-site, 2×2 factorial Phase II RCT that examines the synergistic effects of a 6-month ACT intervention on cognition and MRI biomarkers (AD-signature cortical thickness and hippocampal volume) (n=128). In this ACT Trial Blood Biomarkers Study, we will enroll 120 ACT Trial aMCI participants and measure blood biomarkers at baseline, 3, 6, 12 and 18 months. The goals are to: (1) Determine the effect of interventions on blood biomarkers over 6 months; (2) Evaluate blood biomarkers as surrogate endpoints for predicting cognitive responses to interventions over 18 months; and (3; exploratory) Examine blood biomarkers as surrogate endpoints for predicting brain MRI biomarker responses to interventions over 18 months.

Discussion: This study aims to identify new blood biomarkers that can track cognitive decline or AD-related brain atrophy among aMCI patients subjected to a regimen of aerobic exercise and cognitive training. Findings from this study will drive the further use of blood biomarkers in developing effective prevention and treatment strategies for AD dementia.

Trial registration number: This study is an ancillary study of the ACT Trial which was registered with
ClinicalTrials.gov, NCT03313895.

Full Text

Figure 1. Schedule of enrollment, interventions, and blood collections.

Study Period

Pre-intervention

Intervention

Follow up

Timepoint

Enrollment

Baseline

3 months

6 months

12 months

18 months

Enrollment

X

Informed consent

X

Interventions

Blood collections

X

X

X

X

X

X

Background
In 2018, 5.7 million living Americans were known to have a diagnosis of Alzheimer’s disease (AD) dementia, and 13.8 million Americans will be affected by 2050 [1]. The economic toll of AD is high, estimated at $277 billion in 2018 and is projected to increase to $1.1 trillion by 2050 (in 2018 dollars) [1]. Up to the present time, no viable therapies exist to prevent or cure AD [2]. A successful intervention that delays AD onset by 5 years could save $89 billion a year in the United States alone [3]. However, there are issues in selecting the appropriate study endpoints for evaluating efficacy of AD-prevention trials because cognitive decline in amnestic mild cognitive impairment (aMCI, the prodromal stage of AD) or preclinical AD takes years or even decades to manifest. Biomarkers could address this problem and are widely used as surrogate endpoints in other conditions (e.g., blood cholesterol for monitoring lipid-lowering therapy in the prevention of cardiovascular diseases) [4].

In AD, only neuroimaging and cerebrospinal fluid (CSF) biomarkers have been proposed as surrogate endpoints, but they are both costly and/or invasive. Blood biomarkers predictive of treatment responses would be more advantageous to neuroimaging and CSF biomarkers due to their broad clinical applications. Three sets of blood biomarkers have been well studied in AD. The first set are blood neuropathological biomarkers (e.g., decreased amyloid beta 42 [Aβ_42] or ratio of Aβ_42/Aβ_40 and increased phosphorylated tau [p-tau]) that differentially reflect AD pathology [5, 6] and associated neurodegeneration [7], which have also been demonstrated to be associated with cognitive impairment or dementia [7, 8]. The second set include blood neurodegenerative biomarkers total tau [t-tau] and neurofilament light (NfL). Both have shown to be promising as a biomarker of neuronal injury [9]. Increased baseline plasma t-tau levels correlated with greater cognitive decline in older adults with MCI over time [10]. NfL has been tested as a surrogate endpoint for treatment responses in a neurological condition (multiple sclerosis) [11, 12]. The third set are blood neurotrophic biomarkers (e.g., brain-derived neurotrophic factor [BDNF], insulin growth factor–1 [IGF–1], and short-chain acylcarnitines [SCACs]) that are found to promote neurogenesis [13, 14] and protect against cognitive decline [15, 16] and incident AD [17, 18]. Higher plasma BDNF levels were linked to better memory, larger hippocampal volume, and reduced risk for AD [13, 17, 19]. A recent study showed that higher plasma levels of IGF binding protein–3 (IGFBP–3), a protein essential for IGF–1
physiological action [20], were associated with decreased risk for dementia [18]. Our own study further linked higher levels of plasma SCACs (DL-carnitine [C0] and valeryl-L-carnitine [C5]) to less cognitive decline over 7 years in older adults with normal cognition [21]. Studies have examined whether changes in these biomarkers are associated with changes in cognition or in brain MRI biomarkers among people with aMCI [10]. Hence, these blood biomarkers have considerable potential as surrogate endpoints for treatment response in aMCI.

Aerobic exercise and cognitive training are promising lifestyle interventions in the context of AD. However, the mechanisms of action between these interventions differ. Aerobic exercise is hypothesized to induce widespread and permanent molecular and cellular changes that underlie both neurodegeneration and neurogenesis [22–24]. In contrast, cognitive training is hypothesized to induce minor brain structural changes but contribute relatively strong functional changes in trained cognitive domains [25–28]. Furthermore, combined aerobic exercise and cognitive training (ACT) may have a synergistic effect on cognition [29]. However, little is known as to if and how the respective or combined effects of aerobic exercise and cognitive training will impact blood neuropathological, neurodegenerative, and neurotrophic biomarkers.

Study Aims

The objective of this study is to investigate blood neuropathological, neurodegenerative, and neurotrophic biomarkers as surrogate endpoints for treatment response to 3 interventions in older adults with aMCI: aerobic exercise; cognitive training; and ACT. The specific aims and hypothesis of the study are described as follows:

Aim 1: Determine the effect of interventions on blood biomarkers over 6 months in aMCI.

Hypothesis 1a: ACT decreases t-tau, p-tau, and NfL and increases Aβ42 [or ratio of Aβ42/Aβ40], BDNF, IGF–1, and SCACs to a greater extent than aerobic exercise or cognitive training alone.

Hypothesis 1b (primary): Aerobic exercise induces favorable changes in three sets of biomarkers, while cognitive training only causes favorable changes in neurotrophic biomarkers.

Aim 2: Evaluate blood biomarkers as surrogate endpoints for predicting cognitive response to interventions over 18 months in aMCI.
Hypothesis 2: Greater favorable changes in blood biomarkers at 3 and 6 months are associated with more favorable cognitive changes at 6 months and at 12 and 18 months, respectively.

Aim 3 (exploratory): Examine the correspondence between changes in blood and MRI biomarkers in response to interventions over 18 months in aMCI. If intervention effects on blood and MRI biomarkers can be correlated, it will provide further evidence that blood biomarkers are valuable surrogate endpoints in aMCI.

Methods
Design
This blood biomarker study is built on the design of the ACT Trial, which is a single-blinded (participants are blinded to their treatment groups), 2-site (University of Minnesota [UMN]; and the University of Rochester [UR]), 2x2 factorial RCT to test the efficacy and synergistic effects of a 6-month ACT regimen on cognition and relevant mechanisms (aerobic fitness, cortical thickness, and functional connectivity in the default mode network) in older adults (age 65 years and older) with aMCI [29]. Details of the ACT Trial have been published [29]. The ACT Trial will randomize 128 participants equally to 4 arms (n = 32 per arm): aerobic exercise (cycling) only; speed of processing (SOP) cognitive training only; ACT; or attention control for 6 months. All participants will then be followed for another 12 months. Cognition, including executive function and episodic memory, will be assessed at baseline, 3, 6, 12, and 18 months. MRI biomarkers (AD-signature cortical thickness and hippocampal volume) will be assessed at baseline, 6, 12, and 18 months. Because the ACT Trial does not collect blood samples, our ancillary study will enroll ACT Trial participants, perform blood collections and measure blood biomarkers at baseline, 3, 6, 12, and 18 months.

Settings
Blood sample collections and processing will be performed using the same standardized protocols at both study sites. All samples will be stored in the -80°C freezer in PI Li’s lab. We will perform biochemical analyses of blood biomarkers at the UMN under PI Li’s supervision. All personnel involved in blood sample collection, processing, storage, and biomarker biochemical assessment will be blinded to participants’ ACT Trial treatment arm assignment.

Study population
Recruitment and consent

Recruitment for this ancillary study will begin as soon as the ACT Trial initiates enrollment of its participants. Participants meeting the ACT Trial eligibility criteria [29] will need to meet 2 additional requirements to be eligible for the ancillary study: (i) agree to donate 20 mL at each blood collection (100mL total); and (ii) agree to fast for at least 8 hours (no food or drink other than water and prescribed medicines) before blood collection. The ACT Trial project managers will recruit and screen participants for eligibility and consent to the ancillary study. We will only enroll participants who have consented to be in this study. We will be blinded to participants’ treatment arm in the ACT Trial, and we will not seek to balance the participants in the blood biomarker study in terms of ACT Trial treatment arms.

Sample size and power

All 128 ACT Trial participants are expected to meet these two eligibility criteria. We expect to enroll 94% of ACT Trial’s participants to this ancillary study and have the same attrition rates as the ACT Trial: 25% at 6 months, 30% at 12 months, and 35% at 18 months. As a result, our sample sizes will be 120 at baseline, 90 at 6 months, 84 at 12 months, and 78 at 18 months. Our study is a pilot study and will provide preliminary results to estimate effect sizes for power calculations in a future large-scale study. We evaluated power for all 3 aims using a total sample size of 90 at 6 months and uneven distribution of sample sizes across 4 arms (with the difference in sample size between any 2 arms no greater than 3). For Aim 1, we conducted power calculations based on 5,000 Monte Carlo simulations for the primary hypothesis \( (Hypothesis\ 1b) \), which is to test the main effects of aerobic exercise and cognitive training on blood biomarker levels at 6 months. With a sample size of 90, Aim 1 has 86% power to detect a moderate main intervention effect (Cohen’s d of 0.9). Mean plasma NfL levels were decreased by a Cohen’s d of 0.9 in response to drug therapy in a neurological condition (multiple sclerosis)[11]. Therefore, a Cohen’s d of 0.9 is realistic and achievable for neurological conditions such as MCI and AD. For Aim 2, we want to test the hypothesis of associations between blood biomarker changes and cognitive changes over time using linear regression models, which have the cognitive changes as the outcomes, the blood biomarker changes (in log scale), as well as
interventions, age, sex, and a binary indicator for the presence of the APOE e4 allele as the predictors. We calculated powers using the R package ‘powerMediation’. Aim 2 has over 80% power to detect a weak correlation (r = 0.3) at a significance level of 0.05. For exploratory Aim 3, we will have the same power as for Aim 2.

Retention
The ACT Trial will use 16 retention and adherence strategies: 10 at the program level to improve safety, enjoyment, comfort, and convenience, and 6 at the staff level to enhance communication, rapport, sympathy, and encouragement [29]. We further contribute to recruitment, enrollment, retention and adherence in both studies by employing 5 additional strategies to address the procedural concerns: training skilled phlebotomists for blood collection to ensure safety and comfort; conducting phone calls after blood collection to address concerns and ensure future participation; utilizing flexible scheduling of blood collection; providing transportation to blood collection; and compensating participants for blood collection.

Assessment of blood biomarkers

Blood sample collection and processing

We will collect blood samples after but within 2 weeks as the corresponding cognitive assessments at baseline, 3, 6, 12, and 18 months of the ACT Trial. Importantly, we will implement the following rules in blood collection in order to reduce pre-analytical variations that could affect biomarker levels: (i) collect blood samples after at least 8 hours of fasting (only water and medications are allowed), at the same time in the morning (between 8:30 am and 10 am) [30], and after the participant has been sitting for at least 10 mins; (ii) obtain information on medications, infection, vascular disease conditions and diets [8]; and (iii) collect blood at least 24 hours after the last intervention session [31, 32] at 3 and 6 months, in order to mitigate any effects on biomarkers (e.g., IGF-1 and BDNF) from the last bout of intervention.

The ACT Trial project managers at the UMN and UR will schedule the blood collections. Before collection, the ACT Trial project managers and staff will complete a blood-draw assessment designed to record information (e.g., infection, vascular disease conditions and diets) that may affect blood
biomarkers. The day before the blood collection, the project managers remind participants by phone of their appointments and the fasting requirement. On the day of blood collection, trained phlebotomists will collect blood samples following a venous-blood collection protocol. If a participant has forgotten to fast, the phlebotomist will notify the project managers, who will reschedule the blood draw. The phlebotomist will collect a total of 20 mL of blood, half into a 10-mL plasma (EDTA-treated) tube and the other half into a 10-mL serum tube. At the UMN, blood collections will be performed by trained phlebotomists at the study participants’ home. Upon collection, blood specimens will be stored immediately on wet-ice and transported to PI Li’s lab at the UMN for processing. A lab technician will process and aliquot these specimens according to an established protocol. Briefly, the plasma and serum tubes will be gently mixed and centrifuged in 4°C using a temperature-controlled centrifuge with a Swing out Rotor at 1439 g for 15 mins. The tubes will be removed from the centrifuge immediately after completion. From the plasma tube, up to eight plasma aliquots of 500 μL each will be made; from the serum tube, up to six serum aliquots of 500 μL each will be made; packed cells from each plasma tube will be transferred into a 2 mL aliquot. The aliquoted samples (i.e., plasma, serum and packed cells separated from the plasma) will be stored in a −80°C freezer in PI Li’s lab. At the UR, blood specimens will be performed and processed by staff using the same blood collection and processing protocols and stored temporarily a −80°C freezer locally before being shipped to UMN to PI Li’s lab for long-term storage at UMN.

**Biochemical Analyses**

We will perform biochemical analyses of the following biomarkers: (i) plasma neuropathological and neurodegenerative biomarkers Aβ42, Aβ40, t-tau, p-tau, and NfL; (ii) serum neurotrophic biomarkers BDNF and IGF-1 (IGFBP-3 is included as part of evaluation of IGF-1) [18]; (iii) plasma neurotrophic biomarkers SCACs; (iv) APOE genotypes, at the end of the study to minimize the overall variance in biochemical analyses. The biochemical methods are described as follows:

**Plasma Aβ42, Aβ40, t-tau, p-tau, and NfL**

Simoa is an ultra-sensitive method coupled with the HD-1 analyzer to measure blood protein
biomarkers [33] with high precision [8] and elimination of matrix interferences reported with traditional ELISAs for measurement of $\text{A}_\beta_{42}$ [34, 35]. Simoa assays have been recently used in epidemiological studies to measure blood neuropathological biomarkers [8, 11, 36-38]. We have used a Simoa assay to measure plasma t-tau [10]. In this study, we will use commercially available Simoa assays to measure $\text{A}_\beta_{42}$, $\text{A}_\beta_{40}$, t-tau, p-tau, NfL in plasma samples.

**Serum BDNF, IGF-1, IGFBP-3**

In this study, we will use commercially available ELISAs (R&D Systems, Minneapolis, MN) to measure BDNF, IGF-1, and IGFBP-3 in serum samples [39-41].

**Plasma SCACs**

We have used the Biocrates p180 kits to measure plasma metabolites [21, 42]. In this study, we will use the p180 kits to measure 2 SCACs C0 and C5 in plasma samples.

**APOE genotypes**

We will extract DNA from packed cells stored at -80°C using Puregene® reagents (Qiagen, Germantown, MD) and determine APOE genotypes (2/2, 2/3, 3/3, 3/4, 2/4, or 4/4) using Taqman® SNP Genotyping assays for rs429358 and rs7412 (Life Technologies, Carlsbad, CA).

**Data management**

Quality control samples will be used to verify both accuracy and precision of the biomarker assessments. All the biomarker data along with the data collected as part of the ACT Trial will be stored in the Research Electronic Data Capture (REDCap). REDCap is a secure web interface with data checks during data entry and uploading to ensure data quality, and housed on secure servers operated by the UMN Academic Health Center’s Information Systems.

**Statistical analysis**

The ACT Trial will share data on cognition, AD-signature cortical thickness, and other relevant information (e.g., demographics and medications) with this ancillary study for data analysis. We will perform appropriate transformation of cognition (episodic memory and global cognition) and AD-signature cortical thickness data. We will use Holm’s approach to adjust for the hypothesis testing of the multiple neuropathological, neurodegenerative, and neurotrophic biomarkers considered here,
which will control the family-wise error rate [43]. Despite randomization, it is possible that the ACT Trial intervention groups could differ in important variables (e.g., age, education, medical comorbidities, medication use [collected by the ACT Trial] and APOE genotype). We will compare groups in terms of these variables and adjust for them if significant differences are found. Missing data due to missed collection visits, loss to follow-ups, and dropouts will be recorded and reported. Missing data will not be imputed. The Inverse Probability Weighting method or likelihood-based method will be used for data analysis assuming the missing data are missing at random. Analysis of complete data or using other methods such as the mixture pattern model will be conducted to test the sensitivity of the results to the assumption of missing at random. Although our aims are not focused on age, sex, and APOE genotype, we will consider them as key covariates because they are established risk factors for AD [44]. Thus, we will adjust for these 3 variables as covariates in all analyses to test for significant associations using linear models.

Aim 1: Determine the effects of interventions on blood biomarkers over 6 months in aMCI.

To test Hypothesis 1a and 1b, we will investigate the association between interventions and changes in blood neuropathological or neurotrophic biomarker levels over time. We will use separate linear regression models for testing each hypothesis with the 6-month changes of blood biomarker levels as the outcome variables. Hypothesis 1b is the primary hypothesis. The model for testing the primary hypothesis will include 2 binary indicators, for aerobic exercise and cognitive training, respectively, as the predictors to test the main effects of aerobic exercise and cognitive training. The model for testing Hypothesis 1a will include 2 binary indicators, for aerobic exercise and cognitive training, respectively, as well as their interaction term to test for the synergistic effect of these treatments on blood biomarker level changes, respectively. We will also either include age, sex, and a binary indicator for the presence of the APOE e4 allele as covariates. The p-values from these models will be adjusted across all biomarkers using Holm’s approach.

Aim 2: Evaluate blood biomarkers as surrogate endpoints for predicting cognitive responses to interventions over 18 months in aMCI.

To test the hypothesis in this aim, we will develop longitudinal linear regression models that
investigate the association between changes in blood neuropathological or neurotrophic biomarker levels and cognitive changes over time in response to interventions. These models will have the change from baseline for the cognitive responses as the response variable and will include ACT treatment arm, age, sex, presence of the APOE 4 allele, baseline cognition, and a time-varying blood biomarker (e.g., change in a blood biomarker level for a participant between baseline and 3 or 6 months]) as covariates. We will fit a model for each blood biomarker and use Holm’s method for multiple test adjustment. Any significant association between changes in cognition and in biomarker levels would be clinically useful because biochemical assays are much more sensitive to discern changes in blood biomarker levels than cognitive tests to discern changes in cognition for detecting cognitive treatment effects of interventions.

Aim 3 (exploratory): Examine the correspondence between changes in blood and MRI biomarkers in response to interventions over 18 months in aMCI.

The associations between blood and MRI biomarkers at each time point will be evaluated and 95% CIs will be reported. In addition to this analysis, we will adjust for intervention arms as well as the key biological variables. We will also examine longitudinal linear regression models similar to those used for evaluation of the association between blood biomarkers and cognitive responses in Aim 2, which will test the associations after adjusting for covariates, including ACT treatment arm, age, sex, and presence of the APOE e4 allele.

Discussion

While we have carefully designed this study, we have anticipated potential problems and have identified alternative strategies. Our sample size estimation is based on both a 94% enrollment rate and the same attrition rates as the ACT Trial: 25% at 6 months, 30% at 12 months, and 35% at 18 months. Genetic testing and fear of needles are two general concerns that may deter study participants from enrollment into a blood biomarker study. We will train our staff with talking points so that our staff are prepared to alleviate these concerns should they come up in our study. We will closely monitor the enrollment rates at both study sites. We will review key benchmarks such as recruitment, enrollment, blood collection and sample storage each year to identify challenges and
solutions. In the event of lower enrollment rates than anticipated (i.e., 94%), we will learn critical information related to recruitment to guide future studies.

Lifestyle factors (e.g., diets, cognitive activity, and physical activity), medications, and vascular diseases may affect blood Aβ₄₂[8] as well as blood neurotrophic factors [30, 32]. For example, plasma Aβ₄₂ is increased by hypertension, ischemic heart disease, diabetes, medications, and APOE 4 allele.

To control for lifestyle factors, we will conduct a blood-draw assessment before each draw to collect information on several factors, including, but not limited to, medications, infection, and vascular disease conditions, unsupervised physical activity, and strenuous cognitive activity outside the ACT Trial. We will investigate these factors in statistical models and control for them. Furthermore, because we will measure changes in biomarkers in comparison to baseline biomarker levels, each participant’s baseline values will serve as his/her own controls. This approach means that our data analyses will unlikely be affected by medications that are already used and lifestyle factors and conditions that are already manifested at baseline but remain unchanged across the 18-month study period. If medication surveys and the blood-draw assessments indicate that any medications, lifestyle factors or conditions known to affect blood biomarkers have changed dramatically for a participant during the study, we will control for such changes in data analyses.

Studies have shown that transient increases in the plasma neurotrophic biomarkers BDNF and IGF-1 observed following an exercise training session are more dramatic than exercise-induced increases in their resting levels [32]. However, studies have also shown that a program of exercise training also increased resting levels of these biomarkers, and that larger increases correlated with increases in hippocampal volume and memory [45]. It is challenging to incorporate collection of pre- and post-training blood samples into the ACT Trial’s current study design. In addition, because resting levels of neurotrophic factors reflect constant, not just transient stimulation of neurogenesis, and we are interested in how blood neurotrophic factor levels would reflect constant stimulation of neurogenesis, we will evaluate these changes in resting levels of neurotrophic factors.
Unequal dropouts across the 4 arms (3 interventions plus attention control) may produce uneven distribution of sample sizes. The unequal dropouts would affect power calculation in *Aim 1* but not in *Aim 2*, which is the main effect of biomarker. Therefore, we took this into consideration when we calculated power for *Aim 1* by assuming the difference in sample size between any two arms no greater than 3. The uneven distribution of samples sizes across arms does not affect statistical analyses as we include intervention as a covariate in the linear regression models.

**Trial Status**

The status of the cohort study at the time of manuscript submission is open for enrolment. We started enrolment in August 2018, and expect enrolment accrual to complete in May 2022. The manuscript is based on the study protocol version 4.01 (November 11, 2018).

**Abbreviations**

$A\beta_{42}$: amyloid beta 42; $A\beta_{40}$: amyloid beta 40; ACT: Combined Aerobic exercise and Cognitive Training; AD: Alzheimer’s disease; aMCI: amnestic mild cognitive impairment (aMCI); APOE: apolipoprotein E; ARIC: Atherosclerosis Risk in Communities; BDNF: brain-derived neurotrophic factor; C0: DL-carnitine; C5: valeryl-L-carnitine; CI: co-investigator; CSF: cerebrospinal fluid; ELISAs: enzyme-linked immunosorbent assay; MRI: magnetic resonance imaging; NfL: neurofilament light; NIH: National Institute of Health; IGF-1: insulin growth factor–1; IGFBP–3: IGF binding protein–3; IRB: Institutional Review Board; PI: Principal Investigator; p-tau: phosphorylated tau; t-tau: total tau; RCT: Randomized controlled trials; REDCap: the Research Electronic Data Capture; SCACs: short-chain acylcarnitines; UMN: University of Minnesota; UR: University of Rochester.

**Declarations**

**Ethics approval and consent to participate**

This study was approved by the UMN and UR IRBs (UMN IRB#: STUDY00001835, last day of study approval: 10/25/2019; UR IRB#: RSRB00071626, last day of study approval: 07/31/2019). The consent process was detailed in the manuscript. Informed consent are obtained from all study participants.

**Consent for Publication**

*Not applicable.*

**Availability of data and material**

*Not applicable.*
Competing interests
The authors declare that they have no competing interests.

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Authors’ contributions
DL and FY designed the study. DL, FY, FVL supervised all aspects of the study implementation. LZ and CR designed statistical analysis plans. All authors contributed to the draft of the manuscript. All authors read and approved the final manuscript.

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Figures

![Figure 1](image)

Figure 1

Schedule of enrollment, interventions, and blood collections.

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