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**RESEARCH PAPER**

# Plasma metabolomic markers underlying skeletal muscle mitochondrial function relationships with cognition and motor function

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## Abstract

**Background:** Lower skeletal muscle mitochondrial function is associated with future cognitive impairment and mobility decline, but the biological underpinnings for these associations are unclear. We examined metabolomic markers underlying skeletal muscle mitochondrial function, cognition and motor function.
**Methods:** We analysed data from 560 participants from the Baltimore Longitudinal Study of Aging (mean age: 68.4 years, 56% women, 28% Black) who had data on skeletal muscle oxidative capacity (post-exercise recovery rate of phosphocreatine, $k_{PCr}$) via $^{31}P$ magnetic resonance spectroscopy and targeted plasma metabolomics using LASSO model. We then examined which $k_{PCr}$-related markers were also associated with cognition and motor function in a larger sample ($n = 918$, mean age: 69.4, 55% women, 27% Black).
**Results:** The LASSO model revealed 24 metabolites significantly predicting $k_{PCr}$, with the top 5 being asymmetric dimethylarginine, lactic acid, lysophosphatidylcholine a C18:1, indoleacetic acid and triacylglyceride (17:1_34:3), also significant in multivariable linear regression. The $k_{PCr}$ metabolite score was associated with cognitive or motor function, with 2.5-minute usual gait speed showing the strongest association ($r = 0.182$). Five lipids (lysophosphatidylcholine a C18:1, phosphatidylcholine ae C42:3, cholesteryl ester 18:1, sphingomyelin C26:0, octadecenoic acid) and 2 amino acids (leucine, cystine) were associated with both cognitive and motor function measures.
**Conclusion:** Our findings add evidence to the hypothesis that mitochondrial function is implicated in the pathogenesis of cognitive and physical decline with aging and suggest that targeting specific metabolites may prevent cognitive and mobility decline through their effects on mitochondria. Future omics studies are warranted to confirm these findings and explore mechanisms underlying mitochondrial dysfunction in aging phenotypes.

**Keywords:** motor, skeletal muscle, cognition, mitochondria, metabolomics, older people

### Key Points

- Plasma metabolomic markers of skeletal muscle mitochondrial function are mainly from amino acid-related, lysophosphatidylcholines (lysoPCs) and phosphatidylcholine (PCs).
- The metabolomic scores of skeletal muscle mitochondrial function are associated with cognition or motor function.
- Lysophosphatidylcholine (LysoPC) C18:1 and cystine are the top upregulated and downregulated metabolites with cognition and motor function.

## Introduction

Mitochondria are energy-producing membrane-bound organelles supporting biosynthesis and degradation processes, including adenosine triphosphate production from the breakdown of energy-rich molecules. Mitochondrial health is critical to the normative functioning of the central nervous and musculoskeletal systems as both have high energy demands [1]. In fact, mitochondrial dysfunction is cross-sectionally associated with cognitive function and mobility as well as predicts cognitive impairment and the rate of mobility decline in the older population. Recent data have shown that lower mitochondrial DNA copy number, estimated from Whole Genome Sequencing, was associated with impaired cognition and neurodegenerative diseases [2–4]. Mitochondrial dysfunction, defined by decreased skeletal muscle oxidative capacity, was associated with lower physical and cognitive function, as well as greater mobility decline and future cognitive impairment [5–8]. However, the biological underpinnings of these relationships remain unclear. By identifying metabolites associated with both mitochondrial function and aging phenotypes, we may gain insight into mechanisms linking mitochondria to age-related functional decline.

Blood metabolomics quantifies hundreds of downstream small molecules and can reveal possible biological processes and pathways related to specific functions or phenotypes. Mass spectroscopy, in conjunction with liquid chromatography, is often used in metabolomic analyses with high sensitivity for metabolites with low levels of detection [9]. Using the metabolomics approach, we found that longitudinal changes in skeletal muscle mitochondrial function are associated with concurrent changes in lysophosphatidylcholines (lysoPCs) which are precursors of cardiolipin, a lipid mainly located in the mitochondrial inner membrane [10]. Whether other metabolite classes are associated with skeletal muscle mitochondrial function has not been explored. Similarly, the relationships of metabolomic markers of mitochondrial function with cognition and motor function are also unknown.

In this cross-sectional study, we aimed to identify metabolomic markers of skeletal muscle mitochondrial function and further examine whether these markers were associated with cognitive and mobility outcomes of interest in community-dwelling adults.

## Methods

### Study population

Participants were drawn from the prospective Baltimore Longitudinal Study of Aging (BLSA), a study with continuous enrollment since 1958 [11, 12]. BLSA participants are community-dwelling adult volunteers who enrol free of cognitive impairment and major chronic conditions. During each visit to the National Institute on Aging Clinical Research Unit, participants undergo a series of health, cognitive and functional assessments over 3 days. Participants return for follow-up visits every 4 years if they are younger than the age of 60, every 2 years between age 60 and 79, or annually after age 80.

To identify metabolomic markers of mitochondrial function, we analysed data from 560 participants (56% women, 28% Black) who had data on both skeletal muscle mitochondrial function and plasma metabolomics at the first concurrent visit. We then examined the associations between specific metabolomic markers and cognitive and motor function measures in a larger sample of 918 participants (55% women, 27% Black) who had metabolomics data and cognitive or motor function measures of interest at the first concurrent visit. Data in this study were collected from April 2013 to December 2019. All participants provided written informed consent at each visit. The BLSA protocol was approved by the Institutional Review Board of the National Institutes of Health.

### Plasma metabolomics

Blood was collected from the antecubital vein of participants between 07:00 and 08:00 after fasting for at least 8 hours and abstention from smoking and exercise, following a standardized protocol [13]. Blood samples were immediately stored at 4°C, centrifuged at 2300 rpm for 15 minutes within 4 hours of collection, then aliquoted and stored at −80°C. Approximately 78 plasma samples were measured per plate, up to five plates per week. Batch effects were minimised through normalisation to target values of quality control samples on each plate [13]. The collection of ethylenediaminetetraacetic acid-chelated plasma is consistent with biomarker study guidelines [14]. Liquid chromatography with tandem mass spectrometry (LC–MS/MS) was used to quantify small molecule plasma metabolites, and flow injection analysis tandem mass spectrometry (FIA-MS/MS) was used for lipids and hexoses. Concentrations of extracted metabolites were measured following a manufacturer's protocol for a 5500 QTRAP mass spectrometer (Sciex, Framingham, MA, USA) using the MxP Quant 500 kit (Biocrates Life Sciences AG, Innsbruck, Austria) [10]. The methods for the MxP Quant 500 assay were validated according to the European Medicines Agency guideline 'Guideline on bioanalytical method validation EMEA/CHMP/EWP/192217/2009 Rev. 1 Corr. 2' [15]. This includes validation parameters of selectivity, determination of the limit of detection (LOD), lower limit of quantification (LLOQ), upper limit of quantification (ULOQ), carry-over, intra- and inter-day accuracy and precision and short- and long-term stability. LC–MS/MS measurements have additional validation for calibration according to the calibration curve. All measurements performed by biocrates with the validated assay were approved according to a clearance protocol based on the accuracy of the calibrators and accuracy and precision of QC samples present on every measured plate. We preprocessed data by excluding metabolites with more than 30% of the values below the limit of detection. We then imputed values below the LOD with half of the minimum value for each

remaining metabolite [16]. Values were transformed using natural logarithms and computed as Z scores. From the original 630 metabolites assessed by the MxP Quant 500 kit, 468 metabolites were analysed in this study of BLSA participants. Supplementary Table 1 lists excluded small molecules below LOD.

### Skeletal muscle mitochondrial function

*In vivo* $^{31}$P-MRS measurements of phosphorous-containing metabolites were obtained from the quadriceps muscles using a 3 T Achieva MR scanner (Philips, Best, The Netherlands) [7, 17].

Participants were positioned supine in the bore of the scanner and were instructed to perform a fast and intense ballistic knee extension exercise to deplete phosphocreatine (PCr) in the quadriceps muscles with minimal acidification to assess maximal oxidative phosphorylation [18]. $^{31}$P-MRS spectra were obtained with a pulse-acquire sequence before, during and after exercise using a 10 cm, flat surface coil (PulseTeq, Surrey, United Kingdom) secured over the vastus lateralis muscle of the left thigh. The exercise was performed until a reduction in PCr peak height of between 33% and 66% was achieved, and the total duration of MR data acquisition was 7.5 min [7].

Spectral processing was performed using jMRUI (version 5.2) [19, 20]. Maximum oxidative capacity was quantified by the post-exercise PCr recovery rate constant, $k_{PCr}$, determined by fitting the time-dependent recovery of PCr peak area. Higher $k_{PCr}$ values indicate higher skeletal muscle mitochondrial function or oxidative capacity. Participants may not be able to complete the protocol due to health-related reasons, such as lower limb pain, inability to perform the coordinated movement of a smooth, fast kicking motion and impaired hearing affecting receiving instructions and subsequent comprehension. Participants with at least 33% PCr peak height depletion during exercise were included in the analysis.

### Cognition and manual dexterity

We examined two cognitive measures as well as manual dexterity that were associated with $k_{PCr}$, including Trail Making Test part A (TMT-A), Digital Symbol Substitution Test (DSST) and Purdue Pegboard dominant hand performance [8]. TMT-A measures attention, visual scanning and psychomotor speed, by having participants connect dots beginning with the number 1 and ending with the number 25 [21]. DSST measures processing speed and executive function, by having participants write symbols under corresponding numbers [22]. The Purdue Pegboard Test measures manual dexterity involving sensorimotor integration [23].

### Mobility/lower extremity motor function

We examined 5 mobility measures, including usual and rapid 6-meter gait speed, 2.5-min usual gait speed, 400-meter walk time and the Health Aging and Body Composition Physical Performance Battery (HABCPPB). The HABCPPB consists of timed chair stands, timed semi-tandem, full-tandem and single-leg static balance measures and timed usual and narrow 6-meter walks to provide an estimate of physical functioning [24]. The 2.5-min usual speed walk and the 400-meter walk time were components of the Long Distance Corridor Walk [25].

### Statistical analysis

To identify metabolomic markers of $k_{PCr}$, we performed linear regression with the Least Absolute Shrinkage and Selection Operator (LASSO) model using the 'caret' R package which addresses potential collinearity among metabolites, adjusted for age, sex, race and PCr depletion. LASSO modelling produces a sparse linear model from a large dataset using penalized regression [26]. LASSO modelling adds penalties for the number of predictors and shrink coefficients to zero. To select the optimal values for the hyperparameters $\lambda$, we performed 10-fold cross-validation repeated 10 times using the 'repeatedcv' function in the 'caret' package. We then performed pathway analysis based on these markers using corresponding IDs from the Human Metabolome Database (HMDB). To examine the robustness of the findings, we performed additional analyses using multivariable linear regression to examine each metabolite's association with $k_{PCr.}$ We also conducted a pathway analysis based on metabolomic markers significant at $P < 0.05$.

Based on the importance of each metabolomic marker retained in the LASSO model, we computed a metabolite score of $k_{PCr}$ for each participant. This score is the sum of standardized metabolite concentration and corresponding regression coefficient from the LASSO model. To understand how metabolomic markers of mitochondrial function were associated with functional outcomes of interest, we first examined the associations between the LASSO metabolite score of $k_{PCr}$ and outcome measures. Then we examined the associations between each metabolomic marker of $k_{PCr}$ and outcomes of interest. Models were adjusted for age, sex and race, and additionally adjusted for education in models of cognition and manual dexterity and additionally adjusted for height in models of mobility outcomes. We additionally adjusted for comorbidities in models predicting $k_{PCr}$ and body mass index (BMI) in models predicting functional outcomes as sensitivity analyses. In all regression models, metabolites were set as independent variables. We also tested the associations of $k_{PCr}$ and $k_{PCr}$-related metabolites with age using multivariable linear regression after adjusting for sex and race. In this exploratory analysis, significance was set at two-tailed $P < 0.05$. Due to multiple metabolomic measures, we also reported a false discovery rate (FDR)-adjusted $P < 0.05$. RStudio version 4.3.1 (Boston, MA) was used for all analyses.

## Results

Participants' characteristics are presented in Table 1. The mean age of those 560 participants with concurrent data

**Table 1.** Participant characteristics

| | Sample with data on $k_{PCr}$ and metabolomics (n = 560) | | Larger sample with data on phenotypes and metabolomics (n = 918) | |
|---|---|---|---|---|
| | Mean ± SD or N (%) as noted | Range (n) | Mean ± SD or N (%) as noted | Range (n) |
| **Demographics** | | | | |
| Age at assessment, years | 68.4 ± 14.8 | 22.4–98.8 | 69.4 ± 14.5 | 22.4–98.8 |
| Women | 311 (56) | - | 501 (55) | - |
| Black | 155 (28) | - | 250 (27) | - |
| 4-year college or above | 481 (86) | - | 782 (85) | - |
| Height, cm | 167.9 ± 9.3 | 143.8–197.3 | 167.8 ± 9.4 | 143.8–197.3 |
| Body Mass Index, kg/m$^2$ | 26.8 ± 4.6 | 17.1–44.8 | 27.3 ± 4.9 | 17.1–53.8 |
| **Skeletal muscle mitochondrial function** | | | | |
| $k_{PCr}$, $s^{-1}$ | 0.0217 ± 0.0059 | 0.0114–0.0541 | - | - |
| PCr depletion, % | 55 ± 11 | 33–91 | - | - |
| **Cognitive function** | | | | |
| TMT-A, sec | 30.4 ± 10.8 | 11.0–95.0 (n = 553) | 31.6 ± 14.2 | 11.0–230.0 (n = 902) |
| DSST, no. of correct symbols | 45.1 ± 13.0 | 10.0–84.0 (n = 540) | 44.2 ± 13.0 | 10.0–89.0 (n = 874) |
| **Manual dexterity** | | | | |
| Pegboard dominant hand performance, average no. of pins from 2 trials | 12.8 ± 2.3 | 5.0–18.5 (n = 548) | 12.6 ± 2.3 | 5.0–19.0 (n = 885) |
| **Mobility function** | | | | |
| Usual 6 m walk speed, m/s | 1.22 ± 0.23 | 0.26–1.90 (n = 552) | 1.18 ± 0.24 | 0.26–1.90 (n = 907) |
| Rapid 6 m walk speed, m/s | 1.82 ± 0.37 | 0.38–3.21 (n = 552) | 1.76 ± 0.39 | 0.38–3.31 (n = 907) |
| HABCPPB | 2.70 ± 0.56 | 0.13–3.83 (n = 552) | 2.60 ± 0.61 | 0.13–3.83 (n = 897) |
| Usual 2.5 min walk speed, m/s | 1.23 ± 0.18 | 0.29–1.75 (n = 557) | 1.20 ± 0.21 | 0.29–2.45 (n = 899) |
| 400 m walk time, seconds | 268 ± 55 | 156–600 (n = 539) | 278 ± 65 | 126–817 (n = 855) |

Note: TMT-A: Trail Making Test Part A, DSST: Digital Symbol Substitution Test, HABCPPB: Health Aging and Body Composition Physical Performance Battery. The $k_{PCr}$ sample of 560 participants were part of the larger sample of 918 participants.

on $k_{PCr}$ and metabolomics was 68.4 years (56% women, 28% Black) (Table 1). Older age was associated with lower $k_{PCr}$ ($r = -0.458$, $P < 0.001$). The larger sample who had concurrent data on cognition, motor function and metabolomics had a mean age of 69.4 years (55% women, 27% Black) (n = 918) (Table 1). Among the larger sample, those who had $k_{PCr}$ data were younger and had higher performance on cognitive and motor function compared to those who did not have $k_{PCr}$ data (n = 560 versus n = 358) (all $P < 0.05$).

## Metabolomic markers of skeletal muscle mitochondrial function ($k_{PCr}$)

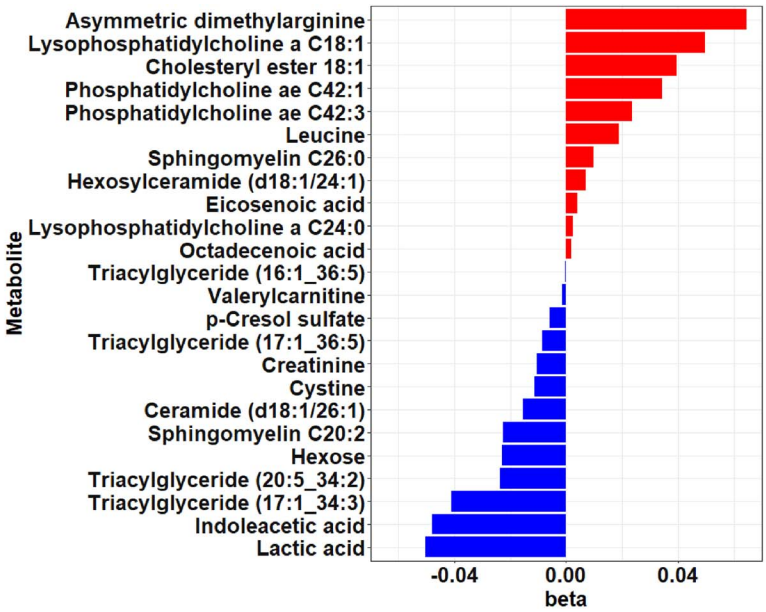
Twenty-four metabolites were retained in the LASSO regression model predicting $k_{PCr}$, including 16 lipids and 8 non-lipids (Figure 1). The $R^2$ from the LASSO model with the optimal lambda was 0.225, explaining 22.5% of the variance in $k_{PCr}$ after adjusting for demographic factors and PCr depletion. The top 10 ranked metabolites were asymmetric dimethylarginine (ADMA), lactic acid, lysoPC a C18:1, indoleacetic acid, triacylglyceride (TG) (17:1_34:3), cholesteryl ester (CE) (18:1), phosphatidylcholine (PC) ae C42:1, TG (20:5_34:2), PC ae C42:3 and hexose, which were also significant in multivariable linear regression at $P < 0.05$ (Figure 1, Supplementary Figure 1). Detailed information on these 24 metabolites, including HMDB ID, pathways\ and findings in the literature are presented in Table 2 and Supplementary Table 2. These 24 metabolites were implicated in five pathways (all $P < 0.05$): sphingolipid metabolism, pyruvate metabolism, glycolysis and gluconeogenesis, galactose metabolism and amino sugar and nucleotide metabolism with sphingolipid metabolism being the top ranked pathway (Figure 1).

In the multivariable linear regression, 110 metabolites were associated with $k_{PCr}$ at $P < 0.05$ after adjusting for demographic factors, and 2 metabolites survived FDR-adjusted $P < 0.05$ (lysoPC a C18:1 and PC ae C42:3) (Supplementary Figure 1, Supplementary Table 3). Sixteen out of these 110 metabolites overlapped with metabolomic markers from the LASSO model, derived from classes TGs (17:1_34:3, 16:1_36:5, 17:1_36:5, 20:5_34:2), lysoPCs (a C18:1, a C24:0), PCs (ae C42:3, ae C42:1), indoles and derivatives (indoleacetic acid), carbohydrates and related (hexose), carboxylic acids (lactic acid), amino acid related (cystine, ADMA), CEs (CE(18:1)), hexosylceramides (d18:1/24:1) and sphingomyelins (SM C26:0) (Supplementary Figure 1). The pathway analysis revealed six significant pathways and four of the six pathways were also identified from the LASSO model, including sphingolipid metabolism, pyruvate metabolism, glycolysis/gluconeogenesis and galactose metabolism (Figure 1, Supplementary Figure 1). After further adjustment for comorbidities, eight metabolites were retained in the LASSO regression model (hexose, cholesteryl ester (18:1), eicosenoic acid, indoleacetic acid, lysoPC a C18:1, phosphatidylcholine aa C42:1, SM

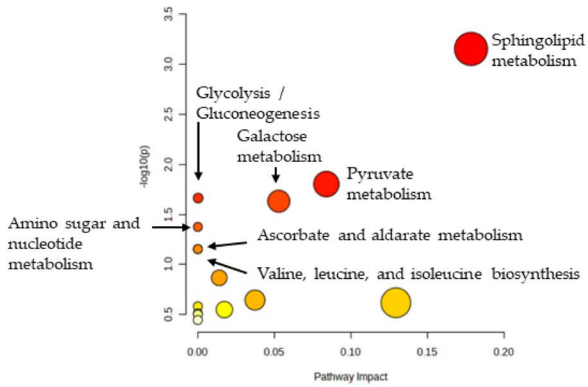
**Table 2.** Significant associations of $k_{PCr}$-related metabolites and corresponding class

| Metabolite names | HMDB | Lipids versus non-lipids | Sub Pathway | Class | Literature refs | beta |
|---|---|---|---|---|---|---|
| Asymmetric dimethylarginine | HMDB0001539 | Non-lipid | Urea cycle; Arginine and Proline Metabolism | Amino Acid Related | CVD, CKD, AD | 0.0646 |
| Lactic acid | HMDB0000190<br>HMDB0001311 | Non-lipid | Glycolysis, Gluconeogenesis, and Pyruvate Metabolism | Carboxylic Acids | Energy, skeletal muscles, brain | −0.0505 |
| Lysophosphatidylcholine a C18:1 | HMDB0002815<br>HMDB0010385<br>HMDB0061701 | Lipid | Lysophospholipid | Lysophosphatidyl-cholines | Cytokines, endothelial cells | 0.0497 |
| Indoleacetic acid | HMDB0000197 | Non-lipid | Tryptophan Metabolism | Indoles and Derivatives | Tryptophan, bacteria | −0.0480 |
| Triacylglyceride (17:1_34:3) | - | Lipid | - | Triglycerides | | −0.0410 |
| Cholesteryl ester 18:1 | HMDB0000918<br>HMDB0005189 | Lipid | - | Cholesteryl Esters | Caudate, putamen, cancer | 0.0394 |
| Phosphatidylcholine ae C42:1 | HMDB0013434<br>HMDB0013447 | Lipid | - | Phosphatidylcholines | Hepatitis B, aging | 0.0343 |
| Triacylglyceride (20:5_34:2) | HMDB0042597<br>HMDB0044273<br>HMDB0047996<br>HMDB0048629<br>HMDB0048651 | Lipid | - | Triglycerides | - | −0.0236 |
| Phosphatidylcholine ae C42:3 | HMDB0013458<br>HMDB0013459 | Lipid | - | Phosphatidylcholines | Insulin resistance, beta amyloid | 0.0236 |
| Hexose | HMDB0000122<br>HMDB0000143<br>HMDB0000169<br>HMDB0000211<br>HMDB0000660<br>HMDB0006088 | Non-lipid | Glycolysis, Gluconeogenesis, and Pyruvate Metabolism | Carbohydrates and Related | Energy, immune cells, diabetes | −0.0228 |
| Sphingomyelin C20:2 | - | Lipid | - | Sphingomyelins | - | −0.0225 |
| Leucine | HMDB0000687<br>HMDB0013773 | Non-lipid | Leucine, Isoleucine and Valine Metabolism | Amino Acids | Lipid synthesis, mitochondria, mTORC1 | 0.0187 |
| Ceramide (d18:1/26:1) | HMDB04954 | Lipid | - | Ceramides | Liver, COVID19, CVD | −0.0156 |
| Cystine | HMDB0000192 | Non-lipid | Methionine, Cysteine, SAM and Taurine Metabolism | Amino Acid Related | Ferroptosis, transport proteins, mTORC1 | −0.0114 |
| Creatinine | HMDB0000562 | Non-lipid | Creatine Metabolism | Amino Acid Related | Skeletal muscle, HF | −0.0105 |
| Sphingomyelin C26:0 | HMDB0011698 | Lipid | - | Sphingomyelins | - | 0.0097 |
| Triacylglyceride (17:1_36:5) | - | Lipid | - | Triglycerides | - | −0.0086 |
| Hexosylceramide (d18:1/24:1) | HMDB0004975<br>HMDB0010712 | Lipid | Hexosylceramides (HCER) | Hexosylceramides | Cancer, hippocampus | 0.0071 |
| p-Cresol sulfate | HMDB0011635 | Non-lipid | Benzoate Metabolism | Cresols | CKD, immune function | −0.0058 |
| Eicosenoic acid | HMDB0002231<br>HMDB0034296<br>HMDB0035159 | Lipid | Long Chain Monounsaturated Fatty Acid | Fatty Acids | Diabetes, oils | 0.0040 |
| Lysophosphatidylcholine a C24:0 | HMDB0010405 | Lipid | Lysophospholipid | Lysophosphatidyl-cholines | | 0.0024 |
| Octadecenoic acid | HMDB0000573<br>HMDB0002080<br>HMDB0003231<br>HMDB0041480<br>HMDB0062218<br>HMDB0062242<br>HMDB0062703 | Lipid | Long Chain Monounsaturated Fatty Acid | Fatty Acids | Diabetes, lipotoxicity | 0.0019 |
| Valerylcarnitine | HMDB0000378<br>HMDB0000688<br>HMDB0013128<br>HMDB0041993 | Lipid | Leucine, Isoleucine and Valine Metabolism | Acylcarnitines | Phenylalanine, muscle breakdown, diabetes, CVD | −0.0015 |
| Triacylglyceride (16:1_36:5) | HMDB0005436<br>HMDB0042323<br>HMDB0042328<br>HMDB0044077<br>HMDB0047922<br>HMDB0048649<br>HMDB0048751<br>HMDB0048758 | Lipid | - | Triglycerides | - | −0.0002 |

Note: CVD: Cardiovascular Disease, CKD: Chronic Kidney Disease, AD: Alzheimer's Disease, HF: Heart Failure. Metabolites are ordered by feature importance. See Supplementary Table 2 for additional literature information.




**(a)**




| Pathway Name | Match Status | p500 Quant (HMDB) Metabolites | *p* | Impact |
|---|---|---|---|---|
| Sphingolipid metabolism | 3/21 | Sphingomyelin (d18:1/26:0), Glucosylceramide (d18:1/24:1 (15Z), Galactosylceramide (d18:1/24:1 (15Z)) | <0.001 | 0.179 |
| Pyruvate metabolism | 2/22 | L-Lactic acid, D-Lactic acid | 0.016 | 0.084 |
| Glycolysis / Gluconeogenesis | 2/26 | D-Glucose, D-Lactic acid | 0.022 | <0.001 |
| Galactose metabolism | 2/27 | D-Galactose, myo-Inositol | 0.023 | 0.053 |
| Amino sugar and nucleotide metabolism | 2/37 | D-Galactose, D-Fructose | 0.042 | 0.0 |
| Ascorbate and aldarate metabolism | 1/8 | myo-Inositol | 0.070 | 0.0 |
| Valine, leucine, and isoleucine biosynthesis | 1/8 | L-Leucine | 0.070 | 0.0 |

**(b)**

**Figure 1.** Feature importance of metabolomic markers of kPCr (a) and pathways involved in these metabolites (b).

C26:0 and triglyceride (17:1_34:3)) which overlapped with metabolites retained in the original LASSO model. In the multivariable linear regression, 69 metabolites were associated with $k_{PCr}$ ($P < 0.05$) which overlapped with significant metabolites in the original multivariable linear regression model (Supplementary Table 3, Supplementary Figure 2).

## Metabolomic markers with cognitive and motor function

LASSO metabolite scores were significantly associated with $k_{PCr}$ ($r = 0.358$, $P < 0.001$) and all cognitive and motor function measures of interest (all $P < 0.001$), except TMT-A ($P = 0.085$) (Figure 2). The association with mobility outcomes appeared stronger than cognitive measures and manual dexterity with correlation coefficients ranging between 0.114 and 0.182 for mobility outcomes and between 0.073 and 0.105 for cognitive measures and manual dexterity (Figure 2).

Among the 24 metabolites retained in the LASSO model of predicting $k_{PCr}$, 17 were associated with cognitive or motor function (Figure 3, Supplementary Table 4). Specifically, 10 metabolites were associated with cognition or manual dexterity, 14 associated with mobility measures and 7 metabolites associated with both cognition, manual dexterity and mobility (Figure 3). Some metabolites from classes carboxylic acids (lactic acid), lysoPCs (a C18:1), PCs (ae C42:3), CEs (18:1), SMs (C26:0) and amino acids (leucine) were all positively associated with cognition or manual dexterity, whereas p-cresol sulfate, cystine, eicosenoic acid and octadecenoic acid were all negatively associated with cognition or manual dexterity (Figure 3). Some metabolites from classes lysoPCs (a C18:1, a C24:0), PCs (ae C42:3, ae

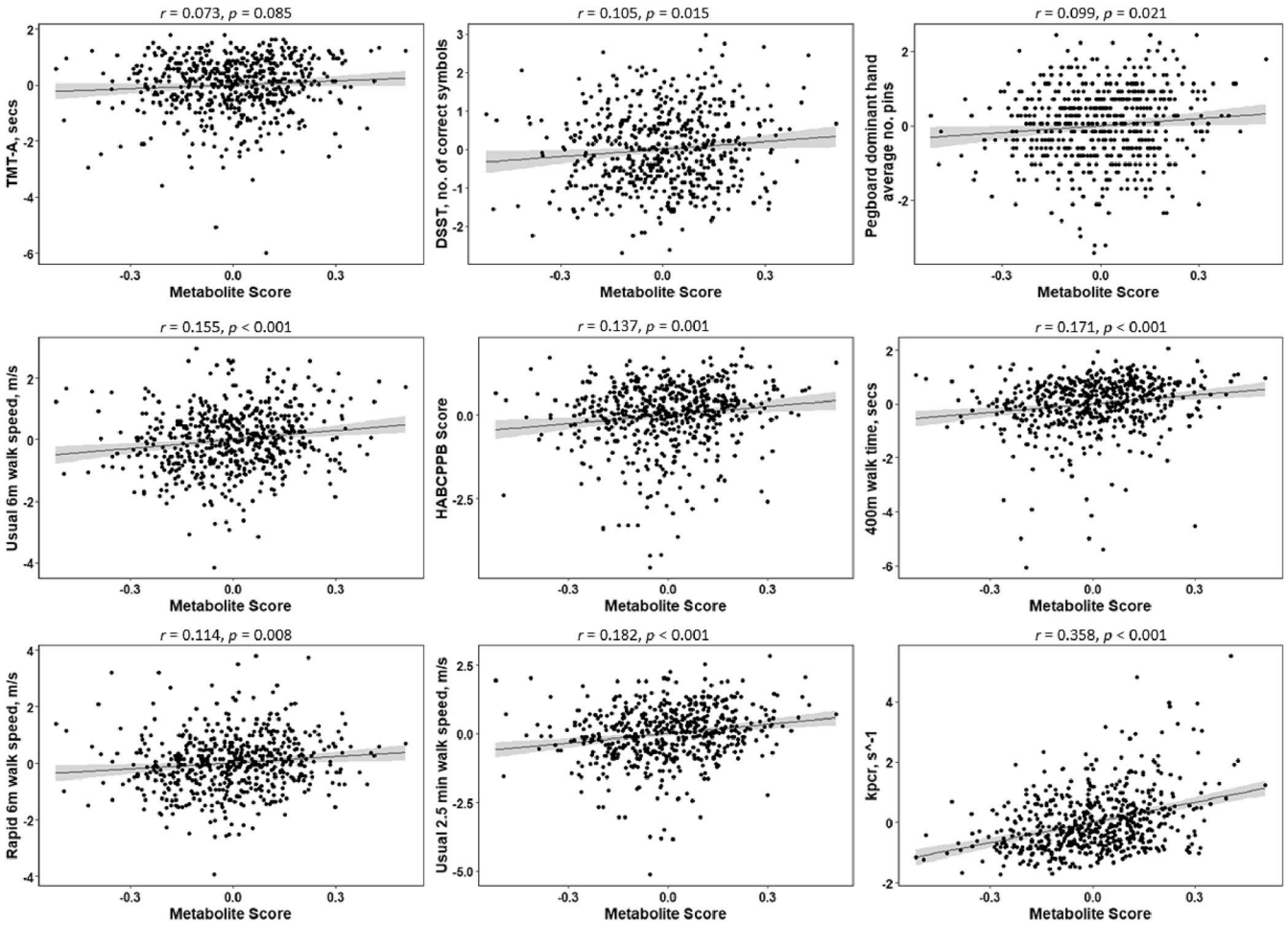


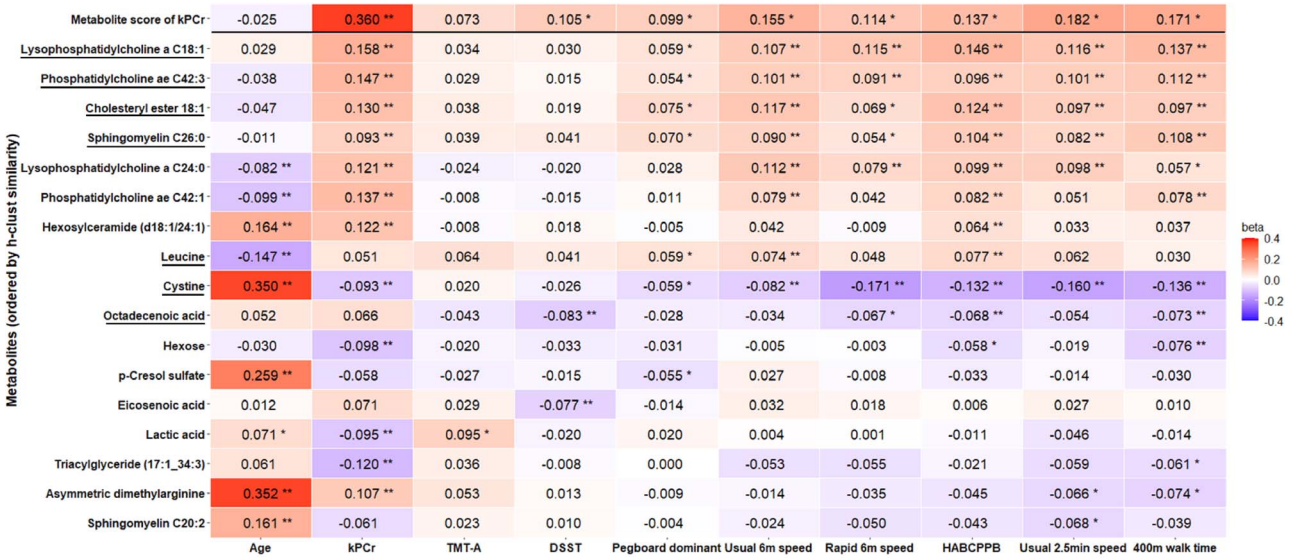

**Figure 2.** Scatterplots of the associations between LASSO metabolite score and cognitive and motor function measures. Legend: all outcome measures are Z scores. Z scores of TMT-A and 400 m walk time were flipped to be consistent with the direction of other measures of interest, as longer times on these two tests are associated with poorer performance, unlike the directional associations of other measures.

C42:1), CEs (18:1), SMs (26:0), amino acids (leucine) and hexosylceramides (d18:1/24:1) were all positively associated with mobility, whereas cystine, octadecenoic acid, ADMA, TG (17:1_34:3), hexose and SM C20:2 were all negatively associated with mobility (Figure 3). Eleven out of the 17 metabolites survived FDR-adjusted $P < 0.05$ (Figure 3). After further adjustment for BMI, results remained similar; 12 metabolites remained significantly associated with at least one cognitive or motor function outcome (Supplementary Table 5). Of these 17 metabolites, some showed positive associations with age after adjusting for sex and race, including hexosylceramide d18:1/24:1, cystine, p-cresol sulfate, lactic acid, ADMA and SM C20:2 (Figure 3a). Some metabolites showed negative associations with age, including lysoPC C24:0, PC ae C42:1 and leucine (Figure 3a).

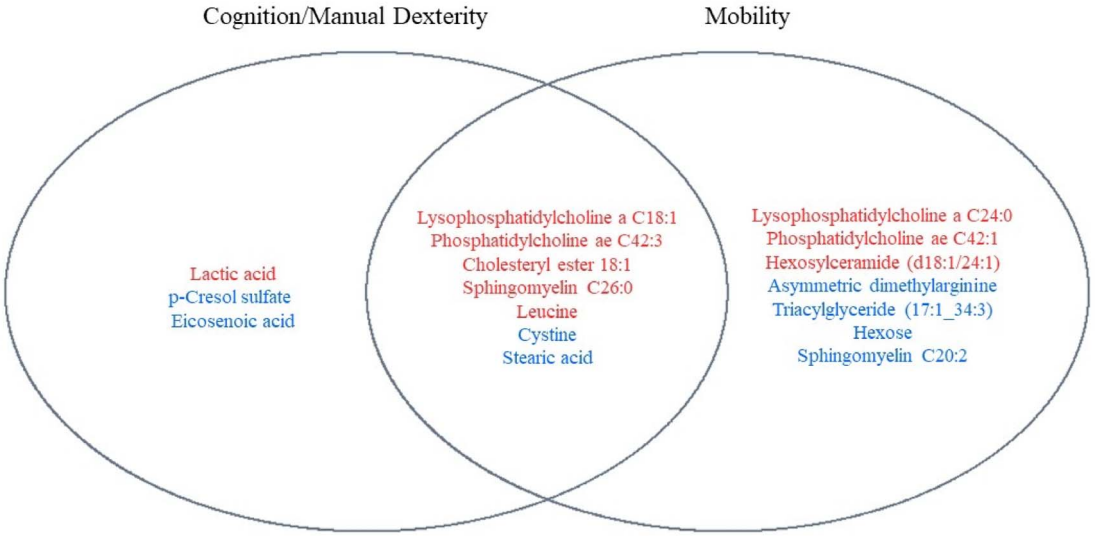
The seven metabolomic markers associated with both cognitive and motor function showed consistent directions (Figure 3, Supplementary Table 3). LysoPC a C18:1, PC ae C42:3, CE(18:1), SM C26:0 and leucine were positively associated with manual dexterity and mobility outcomes, whereas cystine and octadecenoic acid were negatively associated with manual dexterity, processing speed and mobility outcomes (Figure 3, Supplementary Table 3). Notably, octadecenoic acid was associated with mobility measures involving rapid pace, including 6-meter rapid gait, 400 m walk time and HABCPPB (Figure 3). LysoPC a C18:1 and cystine were the metabolites that showed the strongest positive and negative associations with motor function measures, respectively (Figure 3).

## Discussion

In this sample of community-dwelling adults, we identified shared metabolomic markers of skeletal muscle mitochondrial function and cognitive and motor function measures. Novel findings can be summarized in three aspects. First, metabolomic markers of skeletal muscle mitochondrial function were mainly from amino acid-related, carboxylic acids, lysoPCs, indoles and derivatives, triglycerides, CEs, PCs and carbohydrates and related classes, and implicated in five pathways with sphingolipid metabolism being the




**(a)**




**(b)**

**Figure 3.** Heatmap (a) and Venn diagram (b) of kPCr-related metabolomic markers that were significantly associated with cognitive or motor function measures. Legend: Functional outcomes are standardised Z scores. Z scores of TMT-A and 400 m walk time were flipped to be consistent with the direction of other measures of interest. * indicates unadjusted *P*-value <0.05. ** indicates FDR-adjusted $P < 0.05$. In panel a, metabolites were ordered by h-clust similarity of cognitive and motor function only. Metabolites associated with both cognitive and motor function were underlined.

top ranked pathway. Second, the metabolomic scores of $k_{PCr}$ were also associated with cognitive or motor function measures. Third, seven metabolomic markers of $k_{PCr}$, including lysoPC a C18:1, PC ae C42:3, CE(18:1), SM C26:0, leucine, cystine and octadecenoic acid, are associated with both cognitive and motor function in consistent directions.

Specific lysoPCs, including lysoPC a C18:1, were previously identified as biomarkers of skeletal muscle oxidative capacity. We now extended previous research by examining all 26 metabolite biochemical classes [10]. We found 15 additional lipids and 8 non-lipids associated with skeletal muscle oxidative capacity, mainly from classes lysoPCs, PCs and amino acids. Lipids play a major role in mitochondrial function, with PCs and cardiolipin, a derivative of lysoPCs, constituting part of mitochondrial membranes and affecting protein transport in the membrane [27]. Of note, mitochondria are also implicated in the anabolism and catabolism of amino acids. For example, they control leucine degradation and regulation of intracellular cystine levels [28]. These metabolites are implicated in sphingolipid

metabolism, and this finding is in line with previous studies [29]. Sphingolipids are linked to mitochondrial function through lysosomal dysregulation, which alters the lipid composition of the inner mitochondrial membrane and thus energy regulation [30]. Sphingolipids are also involved in dual decline in cognition and mobility [29]. SM C26:0 and hexosylceramide (d1:18/24:1), which are involved in sphingolipid metabolism, may be particularly important for decreased skeletal muscle mitochondrial function as well as cognitive and mobility decline. The associations between $k_{PCr}$-related metabolites and age are also in line with previous findings. For instance, cystine, ADMA and p-cresol sulfate showed strong positive associations with age, while leucine showed a negative association with age [13, 31].

We further expanded prior research by connecting mitochondria-related metabolomic markers to functional outcomes. More than 70% of the metabolomic markers of $k_{PCr}$ are associated with cognition or motor function. When additionally adjusting for BMI, half of the markers of $k_{PCr}$ are associated with cognition or motor function. These metabolites mostly belong to lysoPCs, PCs, CEs, SMs and amino acid-related classes. Seven metabolites, including five lipid and two amino acid-related metabolites, are associated with both cognition and motor function. Findings on lysoPC a C18:1 are consistent with previous studies which reported that lysoPC a C18:1 is associated with skeletal muscle mitochondrial function, cognition and mobility [10, 32–34]. Findings on leucine are also in line with prior literature suggesting it is associated with gait speed, but we expanded these findings by demonstrating that leucine was also associated with cognition [29]. Notably, the metabolites with the strongest association weight, such as lysoPC a C18:1 and cystine, were associated with all mobility outcomes as well as results of the pegboard dominant hand test, a measure of manual dexterity involving sensorimotor integration. These may suggest that metabolite markers of skeletal muscle mitochondrial function are strongly related to motor outcomes.

This study has limitations. First, the study population tends to be healthier due to inclusion criteria and eligibility for the skeletal muscle oxidative capacity study which requires substantial lower extremity mobility and endurance. Second, the sample size of participants with skeletal muscle oxidative capacity data is modest. Future studies with larger and independent samples are needed to confirm these findings. Third, the cross-sectional study design does not allow inferences about causation or address longitudinal trends in the outcome measures. This study has several strengths. First, the state-of-the-art assessment of skeletal muscle oxidative capacity via MR spectroscopy is non-invasive and performed *in vivo*. In addition, our methods used targeted metabolomics, allowing us to examine over 400 metabolites from 26 biochemical classes. Also, the analytical approaches we employed, least squares regression and LASSO modelling, provide complementary findings of metabolites at the individual and group levels. Finally, the inclusion of several cognitive and motor function measures allows for the identification of which cognitive or motor function measures are strongly associated with mitochondria-related metabolites.

In conclusion, the main metabolomic markers of skeletal muscle mitochondrial function belong to amino acid-related, carboxylic acid, lysoPCs, indoles and derivatives, triglycerides, CEs, PCs and carbohydrates and related classes with sphingolipid metabolism being the top ranked significant pathway. The majority of these markers are related to cognition or motor function, with lysoPC a C18:1 and cystine being the top upregulated and downregulated metabolites. Future longitudinal studies with larger sample sizes are warranted to confirm these findings and to further elucidate changes in metabolomic markers with both mitochondrial function and aging phenotypes.

**Supplementary Data** Supplementary data mentioned in the text are available to subscribers in *Age and Ageing* online.

**Declaration of Conflicts of Interest:** None.

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