

Metabolomics of Aerobic Exercise in Chronic Stroke Survivors: A Pilot Study

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Background: Understanding the metabolic response to exercise may aid in optimizing stroke management. Therefore, the purpose of this pilot study was to evaluate plasma metabolomic profiles in chronic stroke survivors following aerobic exercise training. *Methods:* Participants (age: 62 ± 1 years, body mass index: 31 ± 1 kg/m², mean \pm standard error of the mean) were randomized to 6 months of treadmill exercise (N = 17) or whole-body stretching (N = 8) with preintervention and postintervention measurement of aerobic capacity (VO₂peak). Linear models for microarray data expression analysis was performed to determine metabolic changes over time, and Mummichog was used for pathway enrichment analysis following analysis of plasma samples by high-performance liquid chromatography coupled to ultrahigh resolution mass spectrometry. *Results:* VO₂peak change was greater following exercise than stretching (18.9% versus -2% ; $P < .01$). Pathway enrichment analysis of differentially expressed metabolites results showed significant enrichment in 4 pathways following treadmill exercise, 3 of which (heparan-, chondroitin-, keratan-sulfate degradation) involved connective tissue metabolism and the fourth involve lipid signaling (linoleate metabolism). More pathways were altered in pre and post comparisons of stretching, including branched-chain amino acid, tryptophan, tyrosine, and urea cycle, which could indicate loss of lean body mass. *Conclusions:* These preliminary data show different metabolic changes due to treadmill training and stretching in chronic stroke survivors and suggest that in addition to improved aerobic capacity, weight-bearing activity, like walking, could protect against loss of lean body mass. Future studies are needed to examine the relationship between changes in metabolomic profiles to reductions in cardiometabolic risk after treadmill rehabilitation.

Key Words: Stroke—metabolomics—exercise—aerobic fitness

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Introduction

Every 40 seconds someone suffers from a stroke, which is a leading cause of long-term disability.¹ Approximately two thirds of stroke survivors have residual neurological deficits,² often leading to a sedentary lifestyle and declines in peak aerobic capacity (VO_2 peak). Compared to age-matched, generally healthy, sedentary counterparts, VO_2 peak may be reduced by as much as 50% in stroke survivors with hemiparesis.³ Lower levels of VO_2 peak are associated with greater risk for metabolic and cardiovascular related morbidity and mortality.⁴ Though aerobic exercise training can counter these deleterious effects of inactivity in stroke by improving peak aerobic capacity (VO_2 peak),^{5,6} pharmacological stress tests are costly⁷ and the majority of older adults are unable to satisfactorily complete a treadmill exercise test as a screening modality.⁸ Therefore, identifying plasma biomarkers that are associated with VO_2 peak and affected by aerobic exercise are of interest.

High resolution metabolomics (HRM) analyses provide an analytic framework for global of endogenous metabolites and can detail important biologic adaptations influenced by lifestyle adaptations.⁹ Recent studies in generally healthy, nonstroke subjects have identified plasma metabolites spanning a wide range of metabolic pathways associated with VO_2 peak, such as tryptophan and γ -tocopherol,¹⁰ and as differentially expressed by aerobic exercise training, including specific amino acids, lipids, and biogenic amines such as serine, glutamate, sarcosine and kynurenine.^{11,12} Further, recent analyses of HRM spectral data have been employed to measure adaptability or irregularity in biologic systems and metabolism. As greater irregularity is an important feature of health,¹³ multifractal analysis of metabolic networks can provide measures of variation in metabolic signals to reflect nutritional status and states of health and disease. These developments may aid the field of rehabilitation science by identifying those who may benefit the most from aerobic exercise interventions, optimally impacting the assessment and management of stroke. In this study, we tested the potential for metabolomics to detect global metabolic differences between 24 weeks aerobic exercise on a treadmill versus a nonaerobic control (whole body stretching) as a basis to differentiate phenotypic profiles in stroke survivors.

Materials and Methods

Recruitment and Screening

Two-hundred forty-six older (>50 years), chronic (>6 months) stroke survivors with mild to moderate hemiparetic gait were recruited from Baltimore, MD and Atlanta, GA through local newspaper advertisements and Veterans Affairs referral networks. This study was approved by the Institutional Review Board for research involving humans at the University of Maryland School of Medicine, Baltimore, MD and Emory University School of Medicine, Atlanta, GA. Written informed consent was

obtained from 49 potential participants. Participants were required to have completed all conventional physical therapy and be capable of walking 3 consecutive minutes on a treadmill at .3 MPH with handrail support. All were sedentary, performing aerobic exercise more than 30 minutes 2 times per week. Baseline evaluation included a medical history and physical examination to exclude those with poorly controlled hypertension, dyslipidemia, or type 2 diabetes mellitus, heart, liver, renal or hematological disease, anemia, orthopedic, and/or medical conditions that would affect their ability to exercise. Fifteen were medically ineligible; thus, 34 underwent baseline testing.

Aerobic Capacity

A peak exertion, clinician-supervised graded treadmill stress test was performed with indirect calorimetry (Quark, Cosmed USA, Chicago, IL), as previously described,¹⁴ to assess cardiac function and aerobic capacity (VO_2 peak). Tests were terminated at the subject's request or when criteria set forth by American College of Sports Medicine were met.¹⁵ Subjects were allowed handrail support, but were instructed to minimize handrail use to that necessary to maintain balance. VO_2 peak was defined as the highest oxygen consumption value obtained in the last minute of exercise. Peak aerobic testing was determine preintervention and repeated after 24 weeks of intervention (postintervention).

High Resolution Metabolomics

Blood was collected in ethylenediaminetetraacetic acid (EDTA)-containing tubes after a 12-hour fast. Following separation, plasma was stored at -80°C until analysis. HRM profiling by LC-MS was completed using established methods described elsewhere.^{16,17} Briefly, thawed samples were treated with acetonitrile (2:1, v/v), with an internal standard mixture, and centrifuged at 4°C , using established procedures.¹⁸ Following protein precipitation, samples were analyzed in triplicate with high-performance liquid chromatography coupled to ultrahigh resolution mass spectrometry (Thermo-Fusion, Thermo Fisher Scientific, Inc., San Diego, CA) using hydrophilic interaction liquid chromatography operated in positive electrospray ionization a 85 to 1275 mass-to-charge (m/z) range. Separation was obtained with over 10 minutes using a formic acid/acetonitrile gradient with the mass spectrometer set to scan from 85 to 1250 mass-to-charge ratio (m/z). A metabolic feature was defined as a specific m/z along with its retention time and associated ion intensity. Data extraction and peak alignment were completed using apLCMS (available at www.sph.emory.edu/apLCMS)¹⁹ with data quality evaluation by xMSanalyzer designed for use with LC-FTMS data.²⁰ Postintervention blood was drawn 36-48 hours after a final exercise or stretching session.

Interventions

Following baseline testing, participants were randomized 2:1 (exercise: stretching) in Atlanta and a 1:1 in Baltimore to 24 weeks of treadmill training or stretching using a block allocation design for age (<65 years versus ≥ 65 years) and self-selected walking speed from a 4-m walk (<.6 m/s versus $\geq .6$ m/s). Participants performed the treadmill training program 3 times per week and the stretching program 2 times per week in a research gymnasium supervised by an exercise physiologist. All participants attended more than 80% of prescribed exercise and stretching sessions.

Exercise Training Group

Protocols were individualized based on each participant's treadmill walking capacity using methods previously described.²¹ In brief, the treadmill training started conservatively with a goal of 15 minutes total duration at 40%-50% maximal heart rate reserve (HRR). Treadmill exercise was advanced weekly, as tolerated, to 50 minutes duration at an intensity of 60%-70% heart rate reserve. Individuals, whom were too physically deconditioned to walk continuously, exercised intermittently for several minutes as tolerated, with interval rests. Participants received general recommendations and education aimed at modifying cardiovascular disease (CVD) risk factors and promoting physical activity to increase free living activity and prevent disease.

Stretching Group

The stretching group participated in whole-body stretching and balance, lasting approximately 50 minutes. Participants also received general recommendations and education aimed at modifying CVD risk factors and promoting physical activity to increase free living activity and prevent disease, as provided to treadmill exercise group.

Statistical Analyses and Bioinformatics

Descriptive statistics are expressed as mean \pm standard error of the mean (SEM). Baseline values were compared using a two-tailed t test for continuous variables or Fisher's exact test for categorical variables and intervention effects were examined with repeated measures ANOVA. A two-tailed *P* value of <.05 was set as significance.

For metabolomics analyses, technical replicates were averaged, log₂-transformed, quantile normalized, and filtered to include only metabolites that were present in more than or equal to 70% of subjects within at least 1 analytical group. Linear models for microarray data (LIMMA)²² was used to determine differentially expressed features at a significance threshold of .2 after Benjamin-Hochberg (e) false-discovery rate adjustment. To minimize type 2 statistical error, at $q = 0.2$, 80% for values are expected to be correct, and 20% are expected to be

false discovery. Group comparisons assess metabolite differences between: (1) baseline and postintervention for treadmill exercise, (2) baseline and postintervention for stretching, and (3) postintervention for treadmill exercise versus stretching. Two-way hierarchical cluster analysis (HCA) of differentially expressed metabolites were performed to identify metabolite and subject clusters with labeling of intervention to facilitate comparisons. Metabolite annotation and pathway enrichment analyses was performed by *Mummichog* software.¹³ *Mummichog* annotates metabolites based on accurate mass *m/z* and uses permeability testing for significant pathway enrichment.

The multifractal analysis decomposes data into subsets characterized by multifractal spectra and partition function as described by Park et al.²³ Differences in plasma metabolic network variability between groups assessed by multifractal indices of Hurst exponent (H), left slope and partition function from analysis of HRM spectral data, are determined by higher value of left slope from the multifractal spectra indicates deviation from monofractality to suggest the biologic system is more adaptive, irregular, and responsive to stress.²³ Unpaired t tests were used to compare partition functions between groups pre and post intervention and between the aerobic exercise and stretching groups postintervention.

Results

Participant Characteristics

Of the 34 stroke survivors that underwent baseline testing, 4 withdrew due to time constraints, 1 withdrew due to recurrent stroke, 1 withdrew due to a foot ulcer, and 3 were excluded due to noncompliance. Thus, data are reported from the 17 treadmill exercise and 8 stretching participants who completed the intervention. The majority of participants were African American (56%), male (80%), and obese (body mass index (BMI): 31 ± 1 kg/m²). Baseline demographic data and clinical characteristics did not differ between groups (Table 1). As anticipated, the improvement in VO_{2peak} was greater in the treadmill exercise group than the stretching group (18.9% versus $-.2\%$; $P < .01$).

Plasma Metabolome

Comparison of the HRM chemical features in plasma of stroke survivors show that of the 10,632 features detected, 276 features were differentially expressed pre versus post treadmill, whereas 248 of the 10,421 detected features were differentially expressed pre versus post stretching as noted at the false-discovery rate $q = .05$ in the Mahattan plot (LIMMA; $P < .05$). This resulted in the differential expression of a total of 567 of the 10,482 detected features when comparing postintervention treadmill exercise to stretching (LIMMA; $P < .05$; Fig 1).

Two-way HCA was performed using the 267 significant metabolites detected in the comparison between baseline

Table 1. Demographics data

	Treadmill exercise (N = 17)		Stretching control (N = 8)	
	Baseline	Post	Baseline	Post
Age (y)	58.1 ± 1.2	-	61.5 ± 1.3	-
Male (%)	82	-	75	-
African American (%)	53	-	63	-
BMI (kg/m ²)	33.4 ± 1.7	33.3 ± 1.6	28.7 ± 1.6	28.3 ± 1.9
VO ₂ peak (ml/kg/min)	16.9 ± 1.1	23.3 ± 1.2*	19.6 ± 1.1	19.5 ± 1.1

Mean ± SEM.

* $P < .01$: Significantly different than baseline.

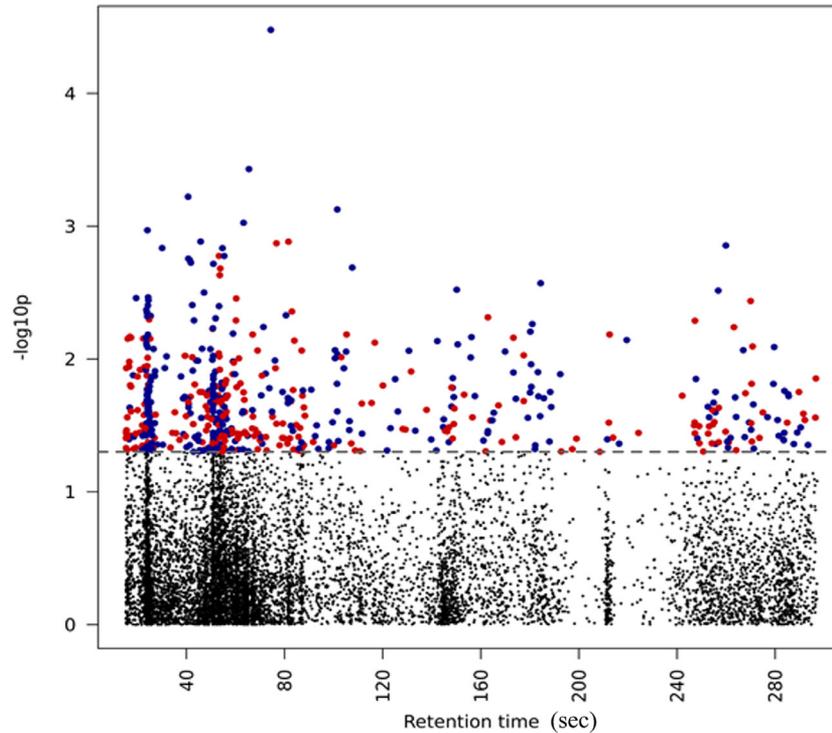


Figure 1. LIMMA analysis discriminates between post treadmill exercise and post stretching. A total of 567 metabolites differed postintervention. Type 2 Manhattan plots showing the negative $\log_{10} P$ value of metabolite comparisons as a function of the chromatographic retention time. Metabolites above the dotted horizontal line were significant at a P value $< .05$. Metabolites are shaded in blue and red by association, positive and negative, respectively, posttreadmill exercise compared to poststretching. Abbreviation: LIMMA, linear models for microarray data.

and postexercise, the 248 significant metabolites between baseline and poststretching and the 576 significant metabolites between postintervention exercise and stretching. HCA showed defined separation between baseline and postexercise for aerobic treadmill exercise and stretching, as well as postintervention between treadmill exercise compared to stretching (Fig 2). *Mummichog* pathway enrichment analyses of differentially expressed metabolites showed that there was enrichment in 4 pathways pre versus post treadmill exercise, 11 pathways pre versus post stretching, and 12 pathways postintervention in treadmill exercise versus stretching (Fig 3). These pathways spanned a variety of metabolic processes and included those related to (1) heparan-, chondroitin-, and

keratan-sulfate degradation and linoleate metabolism, (2) pentose and amino acid metabolism, and (3) amino acid and fatty acid metabolism for post relative to pre treadmill exercise, post relative to pre stretching, and post treadmill exercise relative to post stretching, respectively.

Multifractal analysis of the plasma HRM spectral data characterized by multifractal spectra and partition function showed no differences when comparing preintervention to postintervention for either the treadmill exercise ($P = .26$) or stretching ($P = .37$) interventions. The downward skew of the comparison groups showed evidence of multifractality in all groups. However, the multifractal plot of partition function $T(q)$ showed separation when comparing postintervention data for treadmill exercise

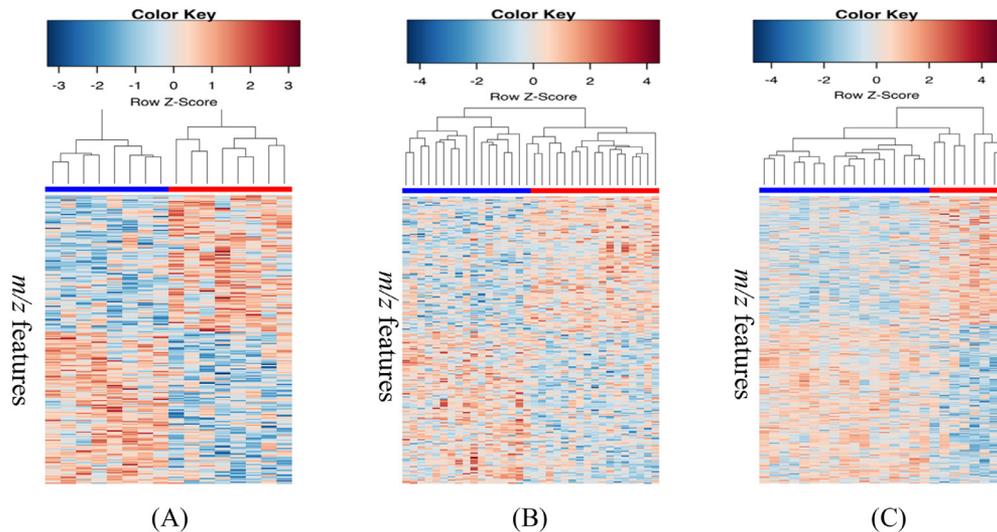


Figure 2. Two-way hierarchical clustering analysis demonstrate the separation by differential expression patterns of features occurring with exercise intervention (A) pre (blue) versus post (red) treadmill exercise, (B) pre (blue) versus post (red) stretching, and (C) post treadmill exercise (blue) versus post stretching (red).

versus stretching (Fig 4). As $T(q)$ approaches $q=0$, the partition function reflects less complexity, thus comparison between treadmill exercise and stretching postintervention that were significantly different in $T(4)$ (treadmill exercise versus stretching: -3.62 versus -3.23 ; $P = .03$) showed less metabolic irregularity and adaptability in the plasma of stroke survivors.

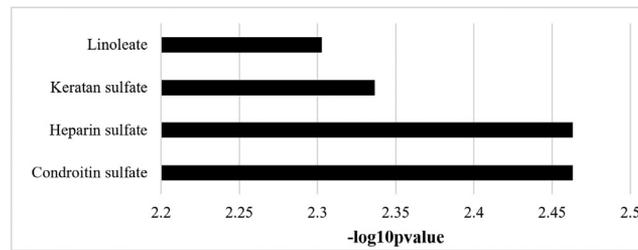
Discussion

Given the abundance of evidence supporting aerobic exercise as a rehabilitation therapy to improve VO_2 peak in stroke survivors,^{5,6} we sought to investigate the effects of treadmill exercise versus a stretching control using a discovery-based plasma HRM approach. Our data reveal that multiple metabolic pathways are significantly influenced by the intervention (i.e. pre versus post) of both treadmill exercise and stretching in individuals undergoing stroke rehabilitation. The HRM measures of the plasma metabolome have sufficient sensitivity to detect multiple pathways altered differentially in response to the treadmill exercise versus stretching. Capturing a broad spectrum of metabolites across a range of chemical classes allows detection of phenotypic differences in metabolic pathways and important biologic features of metabolic irregularity and adaptability resulting from exercise intervention in stroke patients.

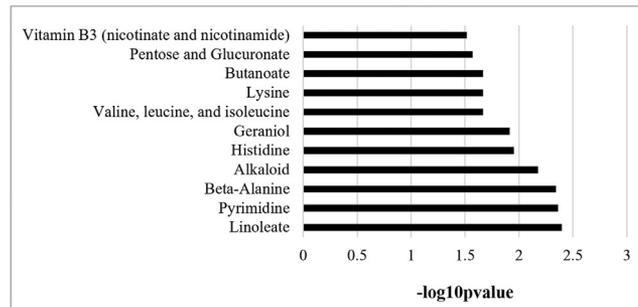
With regard to the effects of the treadmill exercise intervention, we observe changes related to heparan-, chondroitin-, and keratan-sulfate degradation pathways, which may reflect biological activities that are important to reducing stroke risk, including angiogenesis, blood coagulation, and cartilage/collagen formation.²⁴⁻²⁶ Recent evidence from animal models suggests that following an experimental ischemic stroke, treatment with heparan sulfate

proteoglycan and a chondroitin sulfate proteoglycan-degrading enzyme each improved histological (i.e., reduced glial scar thickness) and functional (i.e., reduced forepaw weakness) outcome in the chronic phase of stroke.²⁷ These data suggest that these blood clotting/cartilage formation pathways may be important measures of the effectiveness of rehabilitative therapy. Though these pathways do not overlap with those reported by aerobic exercise training studies in nonstroke populations previously,^{11,12} they support only subtle changes in serum metabolites with treadmill exercise¹² and add to the literature by suggesting that the response to exercise may vary depending upon the population of study. This information may be useful in optimizing rehabilitation recommendations in patients with neurologic-associated disability.

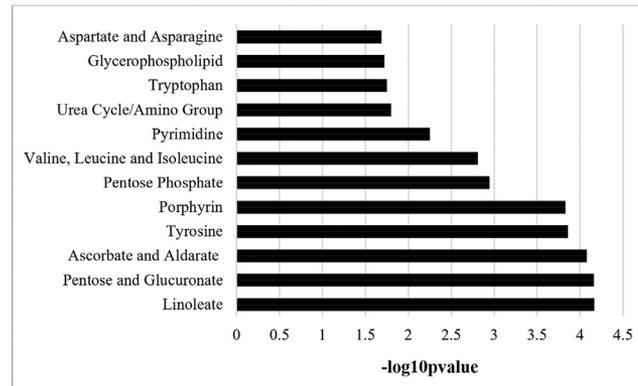
All 3 comparison groups show altered expression of metabolites associated with linoleate metabolism, which is linked to both energy metabolism, mitochondrial function, and cell signaling. The results support past studies that chronic treadmill exercise alters lipid metabolism, which has potential benefit for exercise capacity because lipids are preferred substrates for muscle during endurance-based exercise bouts.²⁸ Though we are unable to identify studies examining the effects of dietary linoleic acid intake on VO_2 peak, studies do not support supplementation with conjugated linoleic acids, isomers of linoleic acid.^{29,30} However, a higher dietary intake of linoleic acid may protect against ischemic stroke.^{31,32} Decreased blood pressure, lipids, glucose, as well as reduced platelet aggregation, and enhanced deformability of erythrocyte cells, are proposed as potential mechanisms of this apparent protective effect.³¹ Nevertheless, because linoleic acid is a precursor of proinflammatory eicosanoids, recommended intakes of linoleic acid are reduced to 5%-10% of total energy intake.³³ These data support previous



(A)



(B)



(C)

Figure 3. Mummichog enriched pathways for metabolites associated with intervention. Metabolic pathway variations comparing (A) pre versus post treadmill exercise, (B) pre versus post stretching, and (C) post treadmill exercise vs. post stretching by mummichog shows total number of pathways altered.

studies³⁴ that regular aerobic exercise by stroke patients may reduce their risk of cardiometabolic complications by impacting lipid metabolism.

Few studies are available to evaluate the effects of non-aerobic, more holistic lifestyle approaches, such as stretching, on metabolomic profiles. Recently, a 6-day intervention designed to assess the effects of herbs, vegetarian diet, meditation, yoga, and massage resulted in a decreased abundance of phosphatidylcholines (70% of all detected metabolites), with the remaining metabolites annotated as amino acids, hydroxy-sphingomyelins, acylcarnitines, and biogenic amines.³⁵ In the current analysis, we observed that pre versus post stretching and the post comparison of treadmill exercise and stretching, had far more differentially affected metabolic pathways than found for pretreadmill and posttreadmill exercise. This was unexpected, and in the next few paragraphs, we will attempt to put these differences into a biochemical context. Importantly, many of the

pathways that changed with stretching also differed in the posttreatment comparison of treadmill and stretching groups, suggesting that the differences are due to time-dependent changes in the stretching group. Loss of lean body mass typically occurs following stroke³⁶ and raises the possibility that the treadmill exercise was more effective in preventing loss of lean body mass than stretching. Of note, treadmill exercise can provide aerobic exercise training comparable to that obtained with a stationary exercise bicycle, but differs in that activity on a treadmill is weight-bearing, while that with an exercise bicycle can be performed with little weight-bearing activity. Decisions for individual patients must be made according to tolerance for weight-bearing activity.

Differentially expressed metabolites for changes with the stretching intervention included pentose, pyrimidine, and glycerophospholipid metabolism and valine, leucine and isoleucine degradation. The pentose phosphate pathway

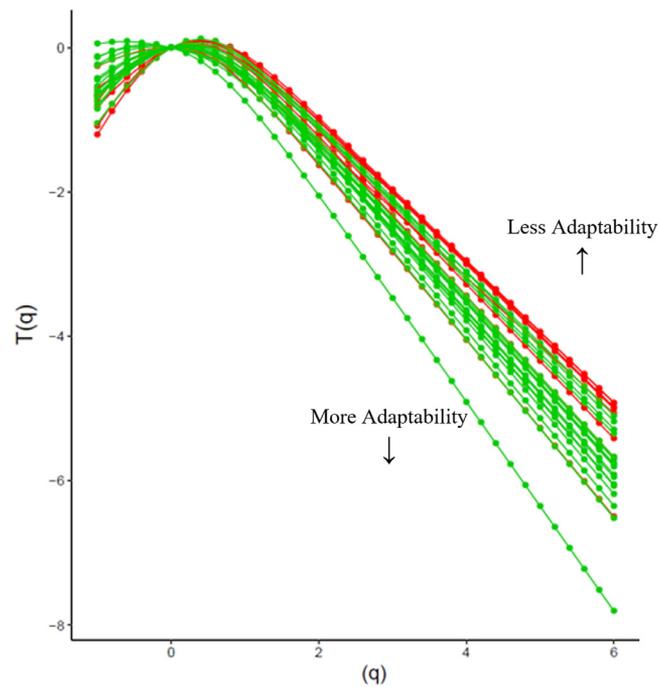


Figure 4. Multifractal spectra plot of partition function ($T(q)$) shows a greater slope for those completing treadmill exercise (green) versus stretching (red) post-intervention (partition function difference in $T(4)$: $P = .03$), indicating that the HRM spectra for the treadmill exercise group is multifractal in greater complexity. Abbreviation: HRM, high resolution metabolomics.

(PPP) produces nicotinamide adenine dinucleotide phosphate, a necessary cofactor for the reduction of glutathione disulfide (marker of reduction in oxidative stress).³⁷ Recent evidence suggests that the PPP plays a key role in regulating vascular function by altering ion channel function, promoting cell proliferation, enhancing cholesterol and fatty acid synthesis, modulating immune system function, and increasing oxidation.³⁸ Also, through its effects on nitrosative stress, which is elevated in chronic stroke,³⁹ the PPP may have a neuroprotective function³⁷ important to stroke survivors. One must be aware, however, that the analyses do not show direction of effects so it is not possible from these data to conclude that the results show a benefit or risk from metabolite changes associated with stretching.

To the best of our knowledge, prior to the current study, evidence relating changes in pyrimidine metabolism to treadmill exercise or stretching interventions had yet to be reported. Three nucleobases found in nucleic acids, cytosine, thymine, and uracil, are pyrimidine derivatives, forming DNA and RNA when paired through hydrogen bonds with their complementary purines.⁴⁰ Pharmacological agents that modulate pyrimidine metabolism are prescribed in neurologic conditions, such as multiple sclerosis, due to their immunosuppressive effects, which appears to delay the progression of disability.⁴¹ However, there is some evidence that they may increase stroke risk by inducing hypertension,⁴² thus their use is not well explored in stroke patients.

Glycerophospholipids not only constitute the backbone of neural membranes, but also provide the membrane with fluidity and ion permeability.⁴³ Though we are

unable to identify previous studies identifying differences in glycerophospholipids metabolism associated with stretching interventions, prior cross-sectional analyses using lipidomic analyses have identified differences in plasma glycerophospholipid concentrations in those with high versus low fitness levels.⁴⁴ Additionally, glycerophospholipid metabolism previously is identified as a potential biomarkers of ischemic stroke,⁴⁵ though there is some evidence from animal models that this relationship may be influenced by obesity.⁴⁶ These data suggest the importance of considering comorbidities when considering the usefulness of biomarkers of stroke.

The 3 proteinogenic branched chain amino acids (BCAAs), valine, leucine, and isoleucine, are among the 9 essential amino acids for humans, accounting for 35% of the essential amino acids in muscle proteins.⁴⁷ Thus, loss of lean body mass in the stretching group could account for the differential effects on this pathway. In addition to playing an important role in muscle protein synthesis, BCAA have diverse physiological and metabolic roles in the body, including an impact on lipolysis, glucose consumption and utilization, and innate and adaptive immune responses,⁴⁷ and thus are extensively used as performance-enhancing supplements. Previous studies have identified changes in the BCAA degradation pathways related to acute and chronic exercise training in younger men.^{48,49} Further, isoleucine is previously proposed as a metabolite biomarkers for acute ischemic stroke.⁵⁰

In this study, we also show that multifractal analysis of the metabolomics data could be useful to discriminate the intervention effects on health and potentially provide a

means to monitor progress within an individual. The concept of fractals provides means to describe complex biological systems based on the irregularity and unpredictability of biologic signals.²³ We show that treadmill exercise results in greater irregularity than stretching, indicating better adaptability. This greater irregularity suggests a greater capability to respond to unpredictable stress, and as such suggests greater health in those completing treadmill exercise.

These data should be interpreted in light of a few limitations. First, our data are acquired from 2 recruitment sites, thus unknown environmental confounders could impact our results. Second, our tissue collection is limited to plasma. Results from animal studies suggest that aerobic exercise training affects the metabolomic profiles of various tissues (i.e. liver versus skeletal muscle) differently.^{51,52} Further, we do not examine these outcomes considering potentially relevant demographic variables, including sex, race, and baseline degree of disability, as this study is not powered to capture this effect. However, this information may be useful to identify those that may receive the greatest benefit from treadmill exercise training and/or stretching interventions. Future controlled studies in larger, diverse samples of stroke survivors are warranted to attempt to replicate our results in plasma and other tissues.

Summary

In summary, findings from the present study advance our understanding of metabolic adaptations to lifestyle interventions in several ways. Our data suggest that attenuation of metabolic response to exercise training may be population specific as they differ from those previously reported in generally healthy adults.^{11,12,48} We find that treadmill exercise affects pathways associated with blood clotting, cartilage formation, and fat metabolism post-stroke. Our data also provide insight into the specific metabolic adaptations associated with treadmill exercise compared to stretching in stroke survivors. Specifically, we observe differences in pathways associated with vascular, neural, and skeletal muscle health that may have an impact on stroke recurrence. These metabolites may be useful as potential biomarkers for the effectiveness of rehabilitation therapy, and may set the stage for the identification of specific metabolic phenotypes that may have the greatest benefit for various lifestyle interventions poststroke.

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Conflict of Interest

None.

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