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Metabolite patterns associated with individual response to supervised exercise therapy in patients with intermittent claudication

Tiffany R. Bellomo, M.D.1*; Noah L. Tsao, B.S.1*; Hillary Johnston-Cox, M.D.2; Kamil Borkowski, Ph.D.3; Gabrielle Shakt, B.S.1,4; Renae Judy, M.S.1; Jonni Moore, Ph.D.5 Sarah J. Ractcliffe, Ph.D.6; Oliver Fiehn, Ph.D.3; Thomas F. Floyd, M.D.7; Felix W. Wehrli, Ph.D.8; Emile Mohler, M.D.9, John W. Newman, Ph.D.3,10,11**; Scott M. Damrauer, M.D.1,4**

* Authors contributed equally to this work
** These authors jointly supervised this work

1 Division of Vascular Surgery and Endovascular Therapy, Perlman School of Medicine, University of Pennsylvania, Philadelphia, PA, USA
2 Division of Cardiovascular Medicine, Perlman School of Medicine, University of Pennsylvania, Philadelphia, PA, USA
3 West Coast Metabolomics Center, University of California Davis, Davis, CA, USA
4 Corporal Michael J. Crescenz VA Medical Center, Philadelphia, PA, USA
5 Department of Pathology, Perlman School of Medicine, University of Pennsylvania, PA, USA
6 Division of Biostatistics, University of Virginia, Charlottesville, VA, USA
7 Departments of Anesthesiology and Pain Management, Cardiovascular Surgery, and Radiology, University of Texas Southwestern, Dallas, TX, USA
8 Department of Radiology, Perlman School of Medicine, University of Pennsylvania, Philadelphia, PA, USA
9 Hospital of the University of Pennsylvania, University of Pennsylvania, Philadelphia, PA, USA
10 Department of Nutrition, University of California, Davis, CA, USA
11 Obesity and Metabolism Research Unit, USDA-ARS-Western Human Nutrition Research Center, Davis, CA, USA

Corresponding Author:
Scott M. Damrauer, M.D.
Division of Vascular Surgery
Hospital of the University of Pennsylvania
3400 Spruce Street, 4 Silverstein
Philadelphia, PA 19104
Tel: 215-615-1698
Fax: 215-614-0463
Email: damrauer@upenn.edu

Keywords: Metabolomics, Peripheral Artery Disease, Lipid Mediators, Supervised Exercise Therapy, Endocannabinoid Signaling
ARTICLE HIGHLIGHTS

Type of Research: Human Study

Key Findings: High levels of change in anandamide (AEA) across SET were associated with a worse response to SET when observing differences in pre- and post-SET baseline metabolomics. Large changes arachidonic acid (AA) and decreased levels of AA precursor dihomo-γ-linolenic acid across SET were associated with a worse response to SET when observing the difference (pre- and post-SET) of the dynamic (pre- and post-treadmill) metabolite changes.

Take home Message: Changes in circulating metabolite patterns associate with individual response to SET. SET may help train patients withstand higher levels of inflammatory signaling and permit them to walk for longer periods of time.
ABSTRACT

Objective: Supervised exercise therapy (SET) is the first line treatment for intermittent claudication due to peripheral arterial disease (PAD). Despite multiple randomized controlled trials proving the efficacy of SET, there are large differences in individual patient’s responses. We used plasma metabolomics to identify potential metabolic influences on the individual response to SET.

Methods: Primary metabolites, complex lipids, and lipid mediators were measured on plasma samples taken at before and after Gardner graded treadmill walking tests that were administered before and after 12 weeks of SET. We used an ensemble modeling approach to identify metabolites or changes in metabolites at specific time points that associated with inter-individual variability in the functional response to SET. Specific time points analyzed included baseline metabolite levels prior to SET, dynamic metabolomics changes prior to SET, the difference in pre- and post-SET baseline metabolomics, and the difference (pre- and post-SET) of the dynamic (pre- and post-treadmill).

Results: High levels of baseline anandamide (AEA) levels pre- and post-SET were associated with a worse response to SET. Increased arachidonic acid (AA) and decreased levels of AA precursor dihomo-γ-linolenic acid across SET were associated with a worse response to SET. Participants who were able to tolerate large increases in AA during acute exercise had longer, or better, walking times both before and after SET.

Conclusions: We identified two pathways of relevance to individual response to SET that warrant further study: AEA synthesis may activate endocannabinoid receptors, resulting in worse treadmill test performance. SET may train patients to withstand higher levels of AA and inflammatory signaling, resulting in longer walking times.
Keywords: Metabolomics, Peripheral Artery Disease, Lipid Mediators, Supervised Exercise Therapy, Endocannabinoid Signaling

INTRODUCTION

Peripheral arterial disease (PAD) is progressive atherosclerotic disease of the aorta and lower extremities that affects more than 200 million people worldwide. Insufficient blood flow to lower extremities leads to ischemia driven symptoms ranging from intermittent claudication to tissue loss. The physical activity limitation of patients with PAD results in functional impairment, loss of mobility, and decreased quality of life.

Supervised exercise therapy (SET) is the first line treatment for individuals with intermittent claudication. Other treatment options for PAD include a combination of supervised exercise therapy (SET), medications, life-style modification such as smoking cessation and weight loss, and revascularization. The efficacy of SET in patients with PAD dates back to 1966 where Larsen et. al demonstrated that 6 months of intermittent walking therapy improved walking time to onset of discomfort and peak walking time (PWT), which represents claudication limited exercise tolerance. There have been multiple clinical trials comparing the benefits of SET with and without revascularization versus optimized medical therapy. However, there has not been consistent evidence for substantial benefit using one intervention strategy over another due to inter-individual variability in response to therapies.

The pathophysiology underlying not only functional decline, but also the effects of exercise observed in patients with PAD, is complex and poorly understood. It is thought that both structural and metabolic disarray in calf skeletal muscle contributes to walking impairment, pain, and functional decline. Although SET does not affect plaque morphology and has
uncharacterized effects of blood flow, there have been several proposed mechanisms of action:

increased calf blood flow via the microvasculature, improved endothelial function and
subsequent improved vasodilatation, reduced inflammation, improvements in muscle structure,
strength and endurance, vascular angiogenesis, improved mitochondrial function, and skeletal
muscle metabolism through lipid-based signaling\(^4\)\(^{,}\)\(^{25-31}\). Despite the fact that metabolomics have
been used to investigate some of these mechanisms\(^32\)\(^ {33}\), metabolomics have not been leveraged
to examine the response to exercise training in patients with PAD.

In this study, metabolomic and lipidomic techniques were utilized to measure the effects
of SET on primary metabolites, complex lipids, and lipid mediators. Through this, we were able
to identify metabolites associated with inter-individual variability in the response of individuals
with PAD to SET. This insight will enhance our understanding of the pathophysiology of lower
extremity symptoms in PAD, the mechanism by which SET improves walking intolerance, and
provide insight on pathways to target for clinical intervention.

METHODS

This study was a secondary analysis of blood samples drawn as part of a clinical trial as
described below. Human subjects protocols were approved by the University of Pennsylvania
Institutional Review Board for and carried out in accordance with relevant guidelines and
regulations. All participants provided written informed consent prior to inclusion in this study.

Study design

The goal of this study was to identify metabolites that were associated with individual
responses to SET. We utilized data and plasma samples from a previously completed randomized
control trial of SET comprised of individuals with peripheral artery disease, as defined by ankle  
brachial indices $\geq 0.4$ and $\leq 0.8$ and the presence of classic claudication symptoms, that were  
randomized to 12 weeks of a standard, validated SET program or usual medical care.\textsuperscript{34}  
12 weeks was selected as the appropriate SET time frame per Clinical Practice Guidelines for standard  
medical treatment of PAD\textsuperscript{35} and all patients who did not undergo SET were excluded from this  
analysis. Prior to initiating and on completion of the exercise program, participants underwent a  
Gardner graded treadmill walking test to determine PWT.\textsuperscript{36} Venous blood was sampled before  
and after each treadmill test using sodium citrate collection tubes, and platelet free plasma was  
stored at $-80^\circ$C (Figure 1).

Individuals who were randomized to the exercise program arm, completed at least 80% of  
prescribed sessions, and had all 4 plasma samples available were selected for metabolic and  
lipidomic analysis. 43 participants in the completed study met these criteria. Based on available  
resources, the 40 participants who completed the greatest number of training sessions were  
selected. This sampling performed within the existing clinical trial lends itself to selection bias of  
who participated in the trial.

**Primary outcome**

The primary outcome was the standardized change in peak walking time (PWT) that  
occurred between the Gardner treadmill test administered before and after SET as a measure of a  
participant’s response to SET. To derive this, a generalized linear model (glm) was constructed  
that modeled the log-transformed PWT (log[PWT]) measured on the post-SET treadmill test  
based on log(PWT) from the pre-SET treadmill test. In the model, the slope ($\beta$) for the pre-  
training log(PWT) represents the average effect of training on the change in log(PWT) across the
cohort. Standardized individual responses are represented by the studentized residuals extracted from the model for each participant. The resultant quantity represents an individual’s personal change in PWT as a result of SET, as compared to the mean group response (Supplemental Figure 1). Negative values represent a less than average increase in PWT as a result of SET and positive values represent a greater than average increase in PWT as a result of SET.

Metabolomic and lipidomic analyses

The plasma samples of the 40 participants were analyzed using one targeted and two untargeted metabolite panels. Primary metabolites were measured on a Leco Pegasus IV, gas chromatography coupled to time-of-flight mass spectrometry (GC-TOF) mass spectrometer with details on sample preparation, derivatization, chromatography parameters, data processing, compound identification and data curation given in Fiehn et al.\textsuperscript{37} Complex lipids were measured on an Agilent 6530 Liquid Chromatography quadrupole time of flight mass spectrometry (LC-QTOF MS) instrument in positive and negative electrospay mode as given in details in Cajka et al.\textsuperscript{38,39} Lipid mediators were separated on a 2.1 x 150mm 1.7µm ethylene bridged hybrid (BEH) C18 column and detected by electrospay ionization with multi reaction monitoring on a API 6500 quadrupole ion trap (QTRAP) and quantified against 7-9 point calibration curves of authentic standards using modifications of previously reported methods.\textsuperscript{40}

Please see Supplemental Methods for detailed methodology.

Data Processing

Any metabolite that was not able to be quantified for every time point for every patient in this small cohort was removed altogether from analysis to prevent any bias introduced by testing.
error and to maintain an adequate sample size for analysis. This resulted in a total of 2507 metabolites with incomplete data across samples removed from analysis. To focus on biological mechanisms, only peaks corresponding to known metabolites were considered in the analysis, for a total of 122 known metabolites in the primary metabolite panel, 306 metabolites in the complex lipids panel, and 64 metabolites in the lipid mediators panel (Figure 2). Raw metabolite levels were natural log transformed and pareto scaled.

Metabolites were annotated with a super class, main class, and sub class by compound name using the RefMet database from the University of California San Diego (UCSD) Metabolomics Workbench (UCSD Metabolomics Workbench). The most specific classification that yielded groups of four or more metabolites in each dataset was used for hypergeometric enrichment. Super class designations were used for the primary and the complex lipids, main class designation was used for the lipid mediators.

**Association testing**

The association of metabolites with inter-individual variability in response to SET was determined using multiple models to maximize discovery while minimizing false positives: repeated measures general linear models (GLM), least absolute shrinkage and selection operator regression (LASSO), elastic net (EN), random forest (RF), and support vector regressions (SVR). The different models each have unique attributes with distinct advantages and limitations: GLM are easy to interpret and computationally efficient but do not perform variable selection and cannot handle situations with more predictors than cases. LASSO and elastic net are modifications of GLM that introduce a penalty variable to perform feature selection and can thus handle situations with more predictors than cases but differ in how they assign the penalty
variable; LASSO eliminates non-informative features while EN does not. Disadvantages include
unstable models due to the requirement to bootstrap data. Random forests is a decision tree
approach that deals well with high dimensional data and is robust to outliers but is
computationally intensive and the effect estimates do not contain information about
directionality. Support vector regression performs well with large numbers of predictor variables
but is not robust to noise in the dataset and does not produce error estimates. By using an
integrative approach of these complementary approaches, we sought to balance the methodical
biases of each approach.42–44

Significant Thresholds

To limit detection of false positives, metabolites of significant interest were defined as
being nominally significant on 2 or more models or experiment wide significant on one model.
For glm, RF, and SVR nominal significance was defined at P < 0.05. For LASSO and elastic net,
metabolites with coefficients not equal to zero were considered to have a nominally significant
association. For all tests, experiment wide significance was defined as a Benjamini-Hochberg
FDR of < 0.05 for the number of known metabolites in the analyzed panel.

Hypergeometric Enrichment

Hypergeometric enrichment was used to identify overly represented classes of
metabolites. The hypergeometric distribution was determined based on the total number of
metabolites, the number of metabolites of interest, and the number of metabolites in the group of
interest calculated using the ‘phyper’ command in base R. Nominal significance was defined as
P < 0.05. Multiple testing correction was performed using a Benjamini-Hochberg false discovery rate (FDR) and experiment wide significance was defined as FDR < 0.05.

RESULTS

Trial Design, Demographic Characteristics, and Response to SET

This study was conducted on a subset of individuals enrolled in a randomized trial of SET. To identify metabolites associated with the inter-individual variability in the response to SET, 40 participants who were randomized to the exercise arm and completed at least 80% of prescribed sessions were selected for metabolite analysis. These participants were a median of 65 years old (IQR 61 - 69) with a median body mass index (BMI) of 28 kg/m² (IQR 26 – 30) and 75% were male (Table 1). The median peak walking times before and after SET were 8.22 (IQR 4.81 – 11.7) minutes and 15.0 (IQR 9.20 – 19.2) minutes respectively. The median change in PWT was relatively modest (5.88 min [IQR 1.65- 10.11 min]), with a large range (1.96 - 35.2 min), suggesting individual level effects of SET training on walking performance. No demographic factors demonstrated an experiment wide significant association with PWT (Table 1). We hypothesized that metabolite levels, static or dynamic, associate with an individual’s response to SET.

To test this hypothesis, we first calculated the individual’s response to SET. We modeled peak walking time on the treadmill test after SET based on peak walking time prior to SET using a generalized linear model. In this model, the slope ($\beta$) of the achieved second treadmill test performance against the predicted second treadmill test performance represents the average effects of SET. To determine individual performance, we calculated the studentized residuals that capture the difference between the predicted and actual peak walking time on the post-SET
treadmill test for each individual. We then tested the participant characteristics for an association
with the studentized residual, or the standardized measure of walking improvement, to identify
confounding clinical factors that contributed to the subject-level variation effects of SET
(Supplemental Figure 1). Although age was nominally associated with the standardized
measure of walking improvement ($\beta = -0.05$, 95%CI = -0.1 to -0.01, p-value = 0.02), no
demographic factors demonstrated an experiment wide significant association with the
studentized residual (Supplemental Table 1).

Baseline and dynamic metabolite levels measured prior to SET associate with
interindividual response to SET

To identify baseline metabolomics pattern prior to SET (Visit 1 Draw 1) associated with
inter-individual differences in the response to SET (Figure 1A), we tested the association of
baseline metabolite levels (Draw 1) from the first study visit with the standardized measure of
walking improvement and identified a total of 32 metabolites of interest (Supplemental Figure
2; Supplemental Table 2). Homogeneous non-metal compounds (p-value = 0.02), sphingolipids
(p-value = 0.05), fatty amides (p-value = 0.04), and sterol and prenol lipids (p-value = 0.03) were
all nominally enriched metabolite classes (Supplemental Table 3) but the significant enrichment
did not persist after correcting for multiple testing.

We then examined whether dynamic changes in metabolite levels (Draw 1 and Draw 2)
that resulted from the Gardner treadmill test prior to the course of 12 weeks of SET (Visit 1)
were associated with inter-individual response to SET (Figure 1B) and identified a total of 28
metabolites of interest (Supplemental Figure 1A; Supplemental Table 4). The metabolite class
enrichment was similar to that seen with baseline metabolites: homogeneous non-metal
compounds (p<0.01, FDR q-value = 0.04), sterol and prenol lipids (p<0.01, FDR q-value = 0.04), and fatty amides (p<0.001, FDR q-value = < 0.01) had experiment wide significant enrichment (Supplemental Table 5). In all cases, the metabolites of interest in these classes were negatively associated with improvement in PWT demonstrating that large changes in these metabolite levels over the baseline treadmill test were associated with diminished response to SET.

Changes in metabolite levels that occur over the course of SET associate with individual response to SET

Changes in pre-exercise metabolite levels that occurred as a result of a 12-week course of SET (Draw 3) were tested against the standardized measure of walking improvement (Figure 1C). We first tested the association of the difference in baseline metabolite levels from plasma samples collected before and after 12-weeks of SET with standardized measure of individual improvement in PWT and identified a total of 73 metabolites of significant interest (Supplemental Figure 1B; Supplemental Table 6) that associated with improvement in PWT. Fatty acyls (p-value = 0.05), homogeneous non-metal compounds (p-value = 0.04), and glycerophospholipids (p-value = 0.05) were nominally enriched in this model (Supplemental Table 7) but failed to reach experiment wide significant enrichment. Organonitrogen compounds (p-value < 0.01, FDR q-value = 0.02) and fatty amides (p<0.01, FDR q-value = 0.02) were enriched at an experiment wide level. In all cases, the metabolites of interest in these classes were negatively associated with performance on the first treadmill test, meaning large increases in these metabolites after SET before an acute exercise stress were associated with diminished
response to SET. These have both been previously shown to change as a result of acute exercise and effect downstream endocannabinoid signaling.\textsuperscript{46}

Of particular interest, we observed that changes in pre-exercise levels of Acyl ethanolamide (AcylEA) metabolites, including anandamide (AEA), linoleoyl ethanolamide (LEA), and adrenolyt-ethanolamide (C22:5n6-ethanolamide; DEA) test that occurred over the course of SET (Draw 1 and Draw 3) (Figure 1C) were all negatively associated with the response to SET (Table 2). In other words, the greater the SET induced increase in pre-exercise levels of plasma AcylEAs, the worse responses to SET. In addition, we saw that SET induced changes in pre-exercise levels of plasma arachidonic acid (AA) were associated with above average response to SET (Table 2, Supplemental Table 6).

We then tested how the impact of SET on the dynamic changes in metabolite levels (Draw 2 minus Draw 1 subtracted from Draw 4 minus Draw 3) that occur over the course of the Gardner treadmill test associates with individual response to SET (Figure 1D). This experiment identified a total of 25 metabolites of significant interest (Supplemental Figure 1C; Supplemental Table 8). Carbohydrates (p-value < 0.01, FDR q-value = <0.001), glycerolipids (p-value = 0.01, FDR q-value = <0.001), and eicosanoids (p-value < 0.01, FDR q-value = <0.001) were experiment wide enriched in this model (Supplemental Table 9).

DISCUSSION

Metabolomics has been performed on patients with PAD to identify metabolites associated with disease progression\textsuperscript{32,33} as well as on a cohorts of healthy and insulin resistant people after exercise.\textsuperscript{47–49} However, metabolomics has not been used to examine the response to exercise training in patients with PAD. We sought to understand metabolic changes at specific
time points with reference to relative improvement in treadmill performance between individuals (Figure 1). To do this, we used five different models to identify metabolites or changes in metabolites at specific time points that associate with either treadmill test performance or inter-individual variability in functional performance after SET. To further understand how classes of metabolites associate with exercise, enrichment analysis was performed. Importantly, we identified metabolites involved in two skeletal muscle pathways of relevance to individual response to SET which warrant further study for potential clinical intervention: AEA signaling and AA synthesis.

**AEA changes are associated with exercise tolerance**

AEA is an organonitrogen compound derived from non-oxidative metabolism\(^\text{50}\) that functions as an endocannabinoid signaling molecule. In skeletal muscle, AEA exerts its effect on muscle metabolism through the membrane-bound G-proteins CB1 and 2 leading to changes in insulin sensitivity, different patterns of glucose uptake\(^\text{51}\) and inhibition of myotube formation.\(^\text{52,53}\) Our data and other literature support the idea that individuals with PAD who perform poorly on treadmill tests following SET have accumulation of inflammatory byproducts resulting in increased endocannabinoid signaling.

Although AEA is generally considered anti-inflammatory, AEA signaling through CB1 receptors is can also be pro-inflammatory.\(^\text{54}\) Changes in circulating AEA and other AcylEAs during exercise have previously been described in healthy participants,\(^\text{55,56}\) where the plasma concentration of AEA following exercise was shown to correlate with endocannabinoid activity. Therefore the effects of exercise on AEA levels can be directly linked to endocannabinoid signaling.\(^\text{57}\) Downstream activity of AEA signaling via the CB1 receptor has been shown to
inhibit myoblast differentiation, expand the number of satellite cells, and stimulate the fast-
muscle oxidative phenotype.⁵⁸ Chronic CB1 receptor stimulation has also been shown to increase
metabolic inflammation and inflammatory byproducts in mice by inducing glucose intolerance.⁵⁹
Even in acute instances, increased CB1 activation impairs skeletal muscle insulin sensitivity,
which decreases glucose uptake in skeletal muscle⁶⁰,⁶¹ and may contribute to an acquired skeletal
muscle myopathy.⁶²,⁶³

In the current study, we found that participants that had large increases in baseline AEA
levels and dihomo-γ-linolenic acid levels as a result of SET did not respond as well to SET
(Table 2). We hypothesize that increased AEA synthesis leads to increased skeletal muscle
endocannabinoid signaling, decreased energy availability, more pain, increased walking
dysfunction, and decreased response to SET. However, additional focused studies are needed to
mechanistically link AEA and endocannabinoid signaling with walking dysfunction in PAD and
the improvements in walking distance associated with SET.

Arachidonic acid and chronic inflammation

The synthesis of AA is another key pathway that can contribute to the inflammatory
response after exercise. AA is a polyunsaturated fatty acid present in phospholipids throughout
the body, but is especially concentrated in skeletal muscle.⁶⁴,⁶⁵ AA generally has pro-
inflammatory and pro-aggregation effects.⁶⁶ In this study, we observed that large increases in
baseline AA as a result of SET were associated with above average response to SET (Table 2).
AA and its downstream metabolites are known to play roles in both the initiation and resolution
of inflammation of several disease states, including obesity, diabetes, and cardiovascular
disease.⁶⁷ The metabolites of AA are generally inflammatory: while cytochrome P450-dependent
epoxygenated AA metabolites are largely anti-inflammatory, the rapid breakdown of these again yields pro-inflammatory signals.\textsuperscript{68} AA derivatives from the lipoxygenase pathways are involved in promoting hyperalgesia and derivatives from the cyclooxygenase pathway are involved in the production of thromboxane A2 and prostaglandins,\textsuperscript{69} which contribute to an inflammatory response. Based on our findings and known AA biology, we hypothesize that SET helps to train individuals with claudication to withstand higher levels of AA and inflammatory signaling, resulting in longer walking times, however additional experiments that investigate the arachidonic acid pathway in a tissue focused manner and metabolites in this pathway are required to prove this.

It is known that PAD patients overall have increased levels of circulating inflammatory biomarkers\textsuperscript{70}. It is also known that patients are instructed to walk through the pain when inflammatory markers are presumably the highest\textsuperscript{71} in order to develop collateral circulation. Therefore, our findings that patients who withstand higher levels of inflammatory signaling may walk for longer periods of time seems plausible\textsuperscript{72}.

\section*{Limitations}

Our results must be interpreted within the context of the limitations of the study design. This study was a secondary analysis of blood samples drawn as part of a clinical trial not original designed to investigate metabolomics and the sampling scheme and sample handling were not designed to optimize sample integrity for metabolomics: non-fasting blood samples were used for analysis and this work did not include participant diet records to control for the impact of diet on the metabolome. Furthermore, anti-inflammatory medication use was not recorded. These limitations in the experimental design would be expected to introduced noise and thus bias our
results to the null. Participants recruited in this cohort were not necessarily reflective of the usual
treatment population of PAD: only 25% of our cohort was actively smoking while the VQI PVI
reports 47% of registrants are active smokers. This could reflect a recruitment bias that results
from differential willingness to participate in a RCT of supervise exercise therapy between
current smokers and current non-smokers. Although no patient demographics collected
associated with the primary outcome, this limits the generalizability of our study. Although this
study did not have a control group, we have considered each participant’s baseline metabolite
levels in our analysis and examined only individual responses to SET; it is possible, however,
that some of the differences observed may be due to fluctuations detected based on repeat
sampling although this would, again, be expected to bias the results to the null. In terms of
metabolite levels, there is controversy in the literature generalizing the role of a singular
metabolite with respect to overall pathway involvement: some metabolites measured are
involved in multiple pathways and have multiple breakdown products with opposing effects.
Therefore, it is hard to clearly define what is and is not known. Finally, we excluded any
metabolite that was incomplete across samples and therefore cannot comment on the significance
of those metabolites. Metabolite panels did not include all metabolites in many pathways of
interest, making it difficult to draw conclusions about the full regulation of the pathway. Future
work is needed to better validate our metabolic patterns found in a larger participant cohort with
an experimental design optimized specifically for metabolomic analysis, including fasting blood
draws, monitored diet status, and records of medication intake.

CONCLUSION

We identified changes in circulating metabolites and metabolite patterns that associate
with individual response to SET. In particular, we found two pathways, AEA signaling, and AA
synthesis associated with individual response to SET which could be areas for future clinical intervention or drug development. Interestingly, both pathways are involved in inflammatory signaling suggesting SET may help participants tolerate increased inflammation produced during exercise, resulting in longer walking times.
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AUTHOR CONTRIBUTIONS

Conceptualization: SMD, TRB, NLT, EM. Methodology: SMD, SR, TRB, NLT. Data generation and processing: SMD, TRB, NLT, KB, GS. Data visualization: TRB, NLT. Formal analysis: NLT, TRB. Project administration: TRB, NLT, SMD, EM. Supervision: SMD, JWN, OF. Writing and crucial editing: SMD, JWN, TRB, NLT, HJC, KB, GS, RJ, SR, OF, TFF, FWW, EM.

DECLARATION OF INTERESTS

S.M.D. receives research support to his institution from CytoVAS and RenalytixA. All other authors have no conflicts of interest to declare.
SUPPLEMENTAL INFORMATION

Data that were used in this study are available on request from the corresponding author (S.M.D.). Code to perform analyses in this manuscript are available from the authors upon request (S.M.D.). Supplemental methods attached describe the metabolomics methodology in more detail.

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Figure and Table Legends

Figure 1. Scheme for defining metabolites of significant interest. Visit #1 included a draw before the first treadmill test (Draw 1) and after the first treadmill test (Draw 2). Visit #2 after SET included a draw before the second treadmill test (Draw 3) and after the second treadmill test (Draw 4). A) Baseline metabolomics pattern prior to SET. Illustration of the association of baseline metabolite levels at Visit 1 measured prior to SET at Draw 1 with interindividual response to SET. B) Dynamic metabolomics changes prior to SET. Illustration of the association of dynamic metabolite levels at Visit 1 measured prior to SET at Draw 1 and Draw 2 with interindividual response to SET. C) Difference in pre- and post-SET baseline metabolomics. Illustration of the association of the difference in baseline metabolite levels from plasma samples collected before (Visit 1 Draw 1) and after (Visit 2 Draw 3) 12-weeks of SET with interindividual response to SET. D) The difference pre-SET (Visit 1) and post-SET (Visit 2) of the dynamic pre-treadmill (Draw 1 and Draw 3) and post-treadmill (Draw 2 and Draw 4). Illustration of the impact of SET on the dynamic changes in metabolite levels that occur over the course of the Gardner treadmill test associates with individual response to SET.

Figure 2. Flowchart of metabolites excluded from analysis. Metabolites in (A) primary, (B) complex lipid, and (C) lipid mediator metabolites were restricted to known metabolites with 0 missing observations. All metabolite levels were individually natural log transformed and pareto scaled prior to all analysis.

Table 1. Demographic characteristics of participants (n = 40) who had plasma samples drawn before and after treadmill testing.

Table 2. Significant results relevant to pathways described. Metabolites were analyzed in relationship to pre-therapy log(peak walking time) and the standardized measure of individual improvement in peak walking time for each of the three data sets using 5 models: general linear model (GLM), elastic net (EN), lasso, random forest (RF), and support vector machine (SVR). Metabolites of significant interest were classified as achieving nominal significance on two or more models or experiment wide significance on one model. Multiple testing correction threshold was set at a false discovery rate of 0.05 for each model and for each of the three data sets. This table includes only significant results of select metabolites.
**Table 1.** Demographic characteristics of participants (n = 40) who had plasma samples drawn before and after treadmill testing.

<table>
<thead>
<tr>
<th>Demographic Characteristics</th>
<th>Overall (N=40)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (years)</strong></td>
<td></td>
</tr>
<tr>
<td>Median [IQR]</td>
<td>65.0 [60.8, 69.3]</td>
</tr>
<tr>
<td><strong>Gender</strong></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>30 (75.0%)</td>
</tr>
<tr>
<td>Female</td>
<td>10 (25.0%)</td>
</tr>
<tr>
<td><strong>BMI</strong></td>
<td></td>
</tr>
<tr>
<td>Median [IQR]</td>
<td>28.3 [25.8, 30.1]</td>
</tr>
<tr>
<td><strong>Tobacco Use</strong></td>
<td></td>
</tr>
<tr>
<td>Never</td>
<td>5 (12.5%)</td>
</tr>
<tr>
<td>Former</td>
<td>19 (47.5%)</td>
</tr>
<tr>
<td>Recently Quit (&lt; 3 mos)</td>
<td>6 (15.0%)</td>
</tr>
<tr>
<td>Current</td>
<td>10 (25.0%)</td>
</tr>
<tr>
<td><strong>Diabetes</strong></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>18 (45.0%)</td>
</tr>
<tr>
<td>No</td>
<td>22 (55.0%)</td>
</tr>
<tr>
<td><strong>Hypertension</strong></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>36 (90.0%)</td>
</tr>
<tr>
<td>No</td>
<td>4 (10.0%)</td>
</tr>
<tr>
<td><strong>Hyperlipidemia</strong></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>32 (80.0%)</td>
</tr>
<tr>
<td>No</td>
<td>8 (20.0%)</td>
</tr>
<tr>
<td><strong>Pre-Training ABI</strong></td>
<td></td>
</tr>
<tr>
<td>Median [IQR]</td>
<td>0.690 [0.605, 0.763]</td>
</tr>
<tr>
<td><strong>Post-Training ABI</strong></td>
<td></td>
</tr>
<tr>
<td>Median [IQR]</td>
<td>0.750 [0.640, 0.870]</td>
</tr>
<tr>
<td><strong>Pre-Training PWT (min)</strong></td>
<td></td>
</tr>
<tr>
<td>Median [IQR]</td>
<td>8.22 [4.81, 11.7]</td>
</tr>
<tr>
<td><strong>Post Training PWT (min)</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Overall (N=40)</td>
</tr>
<tr>
<td>------------------------</td>
<td>----------------</td>
</tr>
<tr>
<td><strong>Median [IQR]</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Change in PWT (min)</strong></td>
<td>4.95 [1.18, 9.47]</td>
</tr>
<tr>
<td><strong>Median [IQR]</strong></td>
<td></td>
</tr>
</tbody>
</table>
Table 2. Significant results relevant to pathways described. Metabolites were analyzed in relationship to pre-therapy log(peak walking time) and the standardized measure of individual improvement in peak walking time for each of the three data sets using 5 models: general linear model (GLM), elastic net (EN), lasso, random forest (RF), and support vector machine (SVR). Metabolites of significant interest were classified as achieving nominal significance on two or more models or experiment wide significance on one model. Multiple testing correction threshold was set at a false discovery rate of 0.05 for each model and for each of the three data sets. This table includes only significant results of select metabolites.

<table>
<thead>
<tr>
<th>Data Set</th>
<th>Metabolite</th>
<th>Model</th>
<th>Outcome</th>
<th>Direction of Association</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipid Mediators</td>
<td>Arachidonic acid</td>
<td>Draw 1</td>
<td>Peak Walking Time</td>
<td>-</td>
</tr>
<tr>
<td>Lipid Mediators</td>
<td>Anandamide</td>
<td>Draw 1</td>
<td>Peak Walking Time</td>
<td>-</td>
</tr>
<tr>
<td>Lipid Mediators</td>
<td>N-oleoylethanolamide</td>
<td>Draw 1</td>
<td>Peak Walking Time</td>
<td>Individual</td>
</tr>
<tr>
<td>Lipid Mediators</td>
<td>Adrenoyl-ethanolamide</td>
<td>Draw 3 - Draw 1</td>
<td>response to SET</td>
<td>Individual</td>
</tr>
<tr>
<td>Lipid Mediators</td>
<td>Arachidonic acid</td>
<td>Draw 3 - Draw 1</td>
<td>response to SET</td>
<td>+</td>
</tr>
<tr>
<td>Lipid Mediators</td>
<td>Anandamide</td>
<td>(Draw 4 - Draw 3)</td>
<td>response to SET</td>
<td>Individual</td>
</tr>
<tr>
<td>Lipid Mediators</td>
<td>Arachidonic acid</td>
<td>(Draw 2 - Draw 1)</td>
<td>response to SET</td>
<td>-</td>
</tr>
</tbody>
</table>
Figure 1. Scheme for defining metabolites of significant interest. Visit #1 included a draw before the first treadmill test (Draw 1) and after the first treadmill test (Draw 2). Visit #2 after SET included a draw before the second treadmill test (Draw 3) and after the second treadmill test (Draw 4). Metabolites were analyzed in relationship to pre-therapy log(peak walking time) and the standardized measure of individual improvement in peak walking time for each of the three data sets using 5 models: general linear model (GLM), elastic net (EN), lasso, random forest (RF), and support vector machine (SVR). Metabolites of significant interest were classified as achieving nominal significance on two or more models or experiment wide significance on one model. Multiple testing correction threshold was set at a false discovery rate of 0.05 for each model and for each of the three data sets. 

A) Baseline metabolomics pattern prior to SET. Illustration of the association of baseline metabolite levels at Visit 1 measured prior to SET at Draw 1 with interindividual response to SET.

B) Dynamic metabolomics changes prior to SET. Illustration of the association of dynamic metabolite levels at Visit 1 measured prior to SET at Draw 1 and Draw 2 with interindividual response to SET.

C) Difference in pre- and post-SET baseline metabolomics. Illustration of the association of the difference in baseline metabolite levels from plasma samples collected before (Visit 1 Draw 1) and after (Visit 2 Draw 3) 12-weeks of SET with interindividual response to SET.

D) The difference pre-SET (Visit 1) and post-SET (Visit 2) of the dynamic pre-treadmill (Draw 1 and Draw 3) and post-treadmill (Draw 2 and Draw 4). Illustration of the impact of SET on the dynamic changes in metabolite levels that occur over the course of the Gardner treadmill test associates with individual response to SET.
Figure 2. Flowchart of metabolites excluded from analysis. Metabolites in (A) primary, (B) complex lipid, and (C) lipid mediator metabolites were restricted to known metabolites with 0 missing observations. All metabolite levels were individually natural log transformed and pareto scaled prior to all analysis.
This manuscript describes the use of metabolomic techniques to measure the inter-individual effects of supervised exercise therapy (SET) in patients with peripheral artery disease (PAD). We identified high levels of AEA are linked to CB1 signaling and activation of inflammatory pathways. This alters energy expenditure in myoblasts by decreasing glucose uptake and may induce an acquired skeletal muscle myopathy. SET may also help participants tolerate increased levels of AA and inflammation produced during exercise, resulting in longer walking times. This data will enhance understanding of the pathophysiology of PAD and the mechanism by which SET improves walking intolerance.