



Pharmacometabolomics reveals a role for histidine, phenylalanine, and threonine in the development of paclitaxel-induced peripheral neuropathy

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Abstract

Purpose Approximately 25% of breast cancer patients experience treatment delays or discontinuation due to paclitaxel-induced peripheral neuropathy (PN). Currently, there are no predictive biomarkers of PN. Pharmacometabolomics is an informative tool for biomarker discovery of drug toxicity. We conducted a secondary whole blood pharmacometabolomics analysis to assess the association between pretreatment metabolome, early treatment-induced metabolic changes, and the development of PN.

Methods Whole blood samples were collected pre-treatment (BL), just before the end of the first paclitaxel infusion (EOI), and 24 h after the first infusion (24H) from sixty patients with breast cancer receiving (80 mg/m²) weekly treatment. Neuropathy was assessed at BL and prior to each infusion using the sensory subscale (CIPN8) of the EORTC CIPN20 questionnaire. Blood metabolites were quantified from 1-D-¹H-nuclear magnetic resonance spectra using Chenomx® software. Metabolite concentrations were normalized in preparation for Pearson correlation and one-way repeated measures ANOVA with multiple comparisons corrected by false discovery rate (FDR).

Results Pretreatment histidine, phenylalanine, and threonine concentrations were inversely associated with maximum change in CIPN8 (Δ CIPN8) ($p < 0.02$; FDR $\leq 25\%$). Paclitaxel caused a significant change in concentrations of 2-hydroxybutyrate, 3-hydroxybutyrate, pyruvate, o-acetylcarnitine, and several amino acids from BL to EOI and/or 24H ($p < 0.05$; FDR $\leq 25\%$), although these changes were not associated with Δ CIPN8.

Conclusions Whole blood metabolomics is a feasible approach to identify potential biomarker candidates of paclitaxel-induced PN. The findings suggest that pretreatment concentrations of histidine, phenylalanine, and threonine may be predictive of the severity of future PN and paclitaxel-induced metabolic changes may be related to disruption of energy homeostasis.

Keywords Metabolomics · Chemotherapy-induced peripheral neuropathy · Paclitaxel · Breast cancer · Nuclear magnetic resonance

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Introduction

Paclitaxel is routinely used for breast cancer treatment; however, approximately 25% of patients experience treatment delays or discontinuation due to the development and progression of paclitaxel-induced peripheral neuropathy (PN) [1]. Paclitaxel-induced PN substantially diminishes patients' quality of life, and up to 80% of affected patients still experience symptoms of neuropathy up to 2 years after treatment discontinuation [2]. Currently, there is no effective strategy to prevent or treat paclitaxel-induced PN, partially due to a lack of a clear definition of PN phenotype and understanding of its underlying pathophysiology [3, 4]. As a result, clinicians do not have reliable tools to predict PN prior to symptoms becoming clinically apparent. This has created a significant unmet need to discover predictive biomarkers of paclitaxel-induced PN that clinicians can use proactively to identify patients at risk of developing severe PN [5], which may also permit the tailoring of paclitaxel dosing regimens to maximize efficacy and minimize toxicity [6].

Previous clinical studies have evaluated the role of antioxidants, amino acids, and vitamins in prevention and/or treatment of chemotherapy-induced PN (acetyl-L-carnitine [7, 8]; glutathione [9–11]; glutamine [12–14]; multivitamin [15]). There is also evidence suggesting that alcohol-induced neuropathy may be related to low vitamin and nutrient levels [16]. Together, these studies imply that PN may result from a complex, multifactorial interaction of small molecules related to vitamin and amino acid metabolism. As such, it is plausible that there is a metabolic basis for the risk of paclitaxel-induced PN, which could be revealed by metabolomics profiling. Metabolomics simultaneously identifies small molecules in a single biological sample. It captures signals from multiple factors (e.g., genetic, environment, microbiome) in aggregate. Pharmacometabolomics, the clinical application of metabolomics, utilizes unbiased profiling to identify metabolic features prior to and during treatment that are related to treatment outcomes [17]. Proof-of-concept studies have demonstrated the potential of pharmacometabolomics to enhance understanding and/or prediction of chemotherapy-induced toxicities [18–20].

Given this scientific rationale and the need to discover predictive biomarkers of paclitaxel-induced PN, we conducted a secondary, proof-of-concept, pharmacometabolomics analysis using whole blood samples collected prior to paclitaxel treatment and after the first treatment dose from patients enrolled on an observational clinical registry (NCT02338115) to identify metabolic profiles associated with the future development of PN.

Methods

Selection and description of participants

The detailed study design and enrolled patients have been previously described [21]. Briefly, sixty female adult patients (> 18 years old) with the diagnosis of stage I–III or oligometastatic breast cancer planning to receive paclitaxel 80 mg/m² 1-h infusions weekly for 12 weeks were prospectively enrolled to an observational clinical registry (NCT02338115). The secondary analysis was approved by the University of Michigan IRBMed (HUM 00086253) and signed informed consent was obtained from each participant. All procedures performed in this study were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Neuropathy assessment

PN was assessed at pre-treatment (BL), just before the end of the first paclitaxel infusion (EOI), 24 h after the first infusion (24H), and prior to each of the subsequent paclitaxel infusion using paper copies of the validated European Organisation for Research and Treatment of Cancer (EORTC) Quality of Life Questionnaire Chemotherapy-Induced Peripheral Neuropathy (CIPN20). The CIPN20 is a patient-reported outcome (PRO) questionnaire that includes 20 questions about symptoms of sensory, motor, and autonomic neuropathy [22]. Items are scored from 1 to 4 with 1 representing “not at all” and 4 representing “very much.” The treating clinicians did not have access to the CIPN20 data at any point during treatment. Paclitaxel is known to cause a primarily sensory neuropathy; therefore, the primary endpoint for this analysis was the 8-item sensory subscale (CIPN8) that quantifies numbness, tingling, and burning/shooting pain in the upper and lower extremities, difficulty walking due to diminished plantar sensation, and temperature sensation [23]. CIPN8 scores were linearly converted to a 0–100 scale with higher scores implying worse neuropathy [24].

Whole blood 1-D-¹H-NMR metabolomics

Whole blood sample collection and processing

Whole blood (WB) samples were collected at BL, EOI, and 24H into Vacutainer® tubes containing sodium heparin as previously described [25]. Following collection, the tubes were placed in an ice water bath and 600 µL of WB was aliquoted into cryovials and promptly stored at (–80 °C).

Following the completion of the study, cryovials were transferred to liquid nitrogen for long-term storage.

1-D-¹H-NMR workflow

Prior to assay, samples were randomly assigned into batches to avoid potential batch effects. At the time of assay, the samples were thawed in an ice water bath and subjected to a 1:1:1 sample/methanol/chloroform precipitation to remove macromolecules [26]. The resulting upper aqueous methanol layers of each sample were dried by lyophilization (Labconco #7382021 at $-105\text{ }^{\circ}\text{C}$ and 0.014mBar) and resuspended in 50 mM sodium phosphate buffer in D₂O (500 μL) with DSS-d6 internal standard Chenomx IS-2 (Chenomx Inc., Edmonton, Alberta, Canada; chemomx.com) of known concentration. The 1-D-¹H-NMR spectrum of each WB sample was acquired at the University of Michigan's Biochemical NMR Core Laboratory on a Varian (now Agilent, Inc., Santa Clara, CA) 11.74 T (500 MHz) NMR spectrometer with a VNMR5 console operated by host software VNMRJ4.0 and equipped with a 5-mm Agilent "One Probe" [27]. The resulting NMR spectra were analyzed using Chenomx NMR Suite 8.3 (Chenomx, Inc.) [28]. Spectral processing, compound identification, and quantification of metabolites were completed and reconciled by three users who were blinded to the samples' identities.

Data and statistical analysis

WB samples from six out of the sixty patients were selected for initial methodology optimization and were excluded from this analysis. Of the remaining fifty-four patients, forty-eight were included in this secondary pharmacometabolomics analysis based on the availability and the quality of the acquired NMR spectra.

Pretreatment metabolome association with PN

The entire pretreatment NMR metabolite dataset (μM) was log transformed and mean centered to achieve a normal distribution using MetaboAnalyst 3.0 (<http://www.metabolanalyst.ca>) [29]. Pearson correlation was performed to examine the association between each pretreatment normalized metabolite concentration value and maximum change in CIPN8 (ΔCIPN8). ΔCIPN8 was calculated as the difference between the highest value of CIPN8 score during treatment and the baseline. Each resulting p value was corrected for multiple comparisons by calculating the false discovery rate (FDR) using the method of Storey [30]. WB metabolites were ranked by ascending FDR and those with an $\text{FDR} \leq 25\%$ were considered as potentially discriminating [31] and were used in the subsequent bioinformatics analysis.

Treatment-induced metabolic changes and their association with PN

The combined pretreatment and first cycle treatment NMR metabolite dataset (μM) was cube root transformed and auto scaled to achieve a normal distribution using MetaboAnalyst 3.0 (<http://www.metabolanalyst.ca>) [29]. The normalized BL value of each metabolite was compared to that at EOI and 24H using a one-way repeated measures ANOVA followed by the Dunnett's multiple comparison test [32]. Each resulting p value was FDR corrected; those with an $\text{FDR} \leq 25\%$ were used in the subsequent bioinformatics analysis. A heatmap of median fold-change was generated to visualize the magnitude of changes in metabolite concentrations. To assess the association between the metabolic change from BL to EOI and ΔCIPN8 , Pearson correlation was performed as described above. Statistical analyses were performed, and figures were generated using Prism 7[®] (GraphPad software), R Studio (version 1.1.423) with ggplot2 package and Path-Visio (version 3.3.0) (<https://www.pathvisio.org>).

Bioinformatics analysis

To assess the metabolic relationship between the metabolites that may be important in the development of PN, WB metabolites with associated Kyoto Encyclopedia of Genes and Genomes (KEGG) ID and $\text{FDR} \leq 25\%$ were used to generate compound–gene–reaction networks in MetScape, a plugin for Cytoscape (<http://www.cytoscape.org/>). MetScape is a free online bioinformatics tool for visualizing and interpreting metabolomics data in the context of human metabolic networks [33].

Results

Patient demographics

Metabolomics data were acquired from 48 patients enrolled in the clinical study (Supplemental Fig. 1). Table 1 shows the demographic and clinical characteristics of these patients. Prior to treatment, the mean (\pm SD) CIPN8 was 0.87 (\pm 2.57). The mean value (\pm SD) of the maximum CIPN8 scores, reported by all patients during 12 weeks of treatment, was 26.91 (\pm 22.36).

Pretreatment metabolome association with PN

Thirty-four metabolites were identified and quantified in all samples. The entire data set can be found at <http://www.metabolomicsworkbench.org/>. Pretreatment histidine, phenylalanine, and threonine concentrations were inversely correlated with ΔCIPN8 ($p < 0.02$; $\text{FDR} \leq 25\%$) (Fig. 1)

Table 1 Demographic and clinical characteristics of 48 patients

Baseline characteristics	<i>N</i> (%) or mean (\pm SD)
Age (years)	52 (\pm 10)
Self-reported Caucasian race	46 (96%)
Body mass index (kg/m ²)	28.8 (\pm 6.0)
Diabetes diagnosis	2 (4%)
Systematic treatment setting	
Neoadjuvant	13 (27%)
Adjuvant	35 (73%)
Systematic treatment regimen ^a	
Prior A/C	45 (94%)
Concurrent H and/or P	27 (56%)
Baseline CIPN8 (0–100)	0.87 (\pm 2.57)
Maximum CIPN8 during treatment (0–100)	26.91 (\pm 22.36)

^aSystemic treatment regimen: A doxorubicin, C Cyclophosphamide, T paclitaxel, H trastuzumab, P pertuzumab

(Supplemental Table 1). A MetScape-generated network shows the metabolic relatedness between histidine, phenylalanine, and threonine (Fig. 2).

Treatment-induced metabolic changes and their association with PN

Paclitaxel induced statistically significant changes in concentrations of 14 metabolites from BL to EOI, from BL to 24H, or both ($\text{FDR} \leq 25\%$) (Fig. 3). The metabolites 3-hydroxybutyrate and 2-hydroxybutyrate (Fig. 3a, b) increased from BL to EOI and then declined from BL to 24H. Pyruvate, alanine, and threonine (Fig. 3c–e) initially decreased from BL to EOI, but were higher than BL at 24H. Phenylalanine,

tyrosine, asparagine, lysine, o-acetylcarnitine, proline, lactate, glutamine, and leucine (Fig. 3f–n) did not change from BL to EOI, but increased from BL to 24H. *p* values and FDR of each metabolite (BL vs. EOI and BL vs. 24H) are listed in Supplemental Table 2. There was no statistically significant association between the change in concentration of any metabolite from BL to EOI and Δ CIPN8 (data not shown).

A heatmap of the median fold-change (Fig. 4a) shows the magnitude of concentration changes of all metabolites. A MetScape network revealed that paclitaxel impacts multiple pathways (Supplemental Table 3). The primary metabolic pathways that incorporate the differentiating metabolites are glycolysis and the tricarboxylic acid (TCA) cycle; these are depicted in Fig. 4b, c.

Discussion

Paclitaxel-induced PN is a debilitating toxicity that substantially affects patient's quality of life. To the best of our knowledge, this is the first pharmacometabolomics study of paclitaxel-induced PN, and it demonstrates that whole blood metabolomics is a viable approach for PN biomarker discovery. Furthermore, we employed a PRO tool to assess patients' PN symptoms, which has been shown to be more reliable compared to clinician-graded NCI CTCAE [34–36]. This innovative analytic approach in conjunction with PRO data provides new insights into the understanding and prediction of paclitaxel-induced PN. Specifically, they implicate an inverse correlation between pretreatment histidine, phenylalanine, and threonine concentrations and Δ CIPN8, suggesting that the initial concentrations of these three metabolites may have predictive value for the severity of

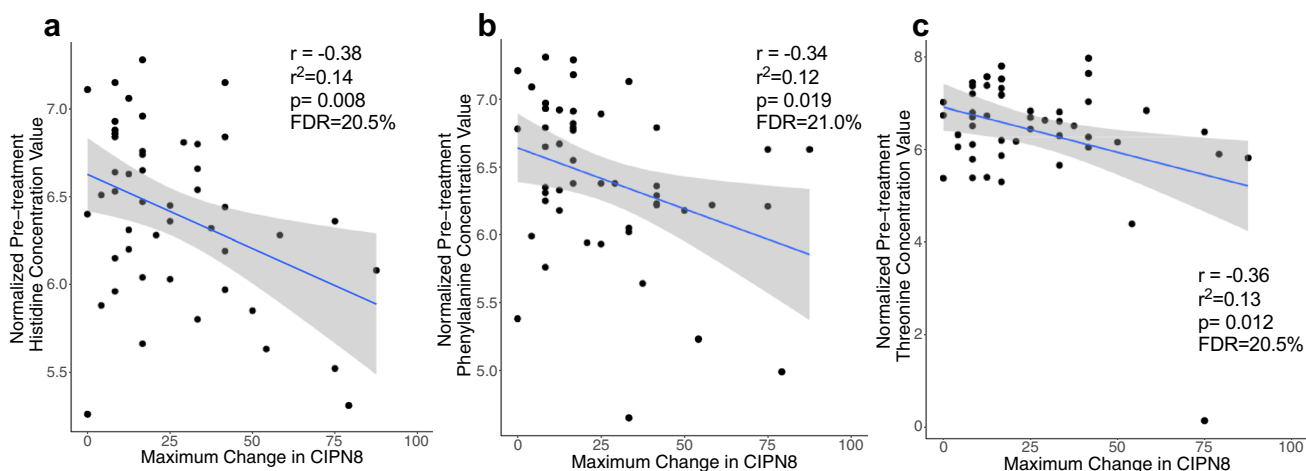


Fig. 1 Pretreatment metabolite concentrations are informative of the development of paclitaxel-induced peripheral neuropathy (PN). Pretreatment concentrations of **a** histidine, **b** phenylalanine, and **c** threonine are inversely correlated with the maximum change in CIPN8

score by Pearson correlation ($p < 0.02$; $\text{FDR} \leq 25\%$). The Pearson correlation coefficient (*r*), r^2 , *p* value, and FDR for each metabolite are shown in each panel (a–c). The blue line represents the fitted line and gray shade represents the 95% confidence interval

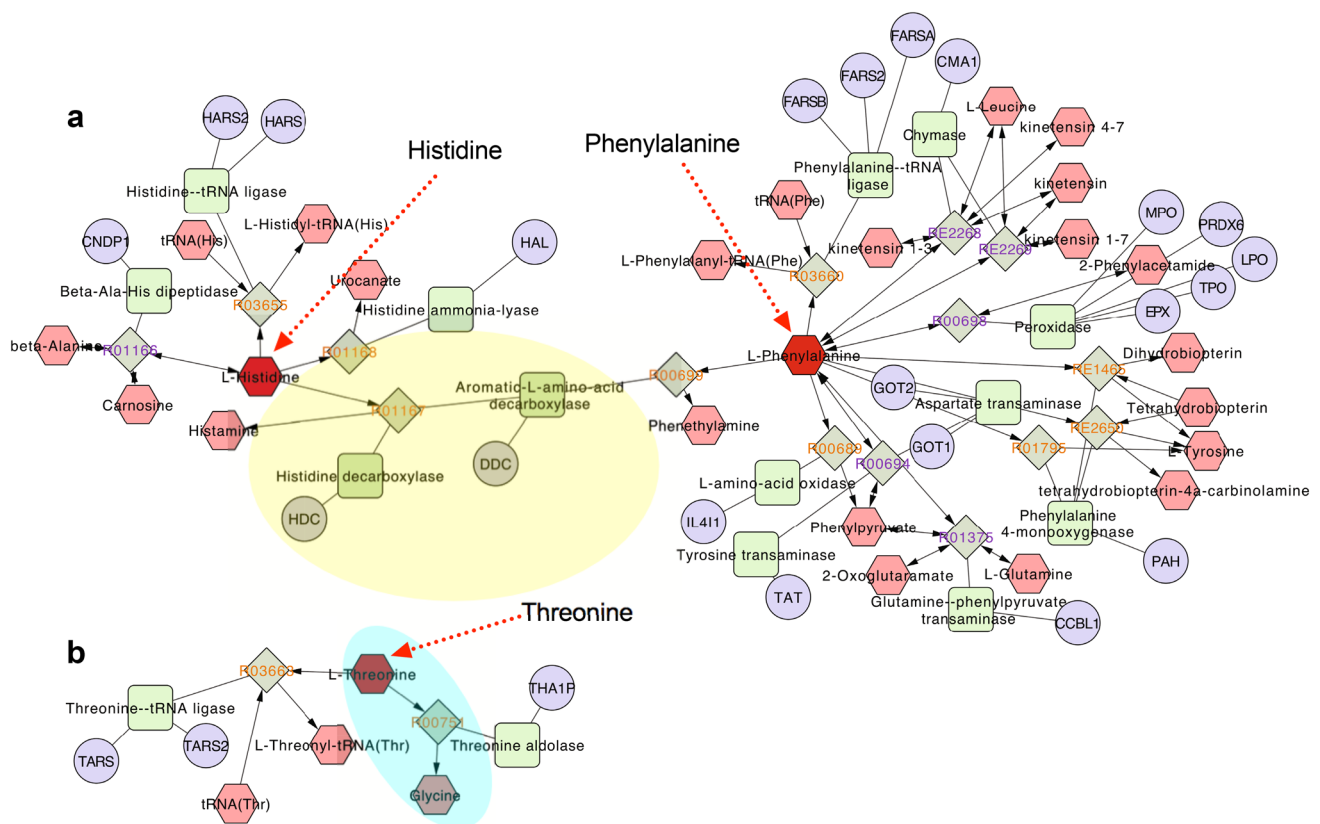


Fig. 2 Metabolic networks of histidine, phenylalanine, and threonine generated by MetScape. Networks illustrate the relationship between uploaded metabolites (input, dark red hexagons): histidine (KEGG ID C00135), phenylalanine (KEGG ID C00079), and threonine (KEGG ID C00188). **a** Both phenylalanine and histidine undergo decarboxylation reactions (R00699 and R01167, respectively), catalyzed by aromatic-L-amino-acid decarboxylase encoded by the DDC gene, to

form phenylethylamine and histamine, respectively (yellow shading). **b** The threonine network is distinct and its primary reaction (R00751) is catalyzed by threonine aldolase to form glycine (blue shading); glycine was not found to be a differentiating metabolite of the development of PN ($p=0.67$; $FDR=99.3\%$). Pink hexagons represent metabolites, green squares represent enzymes, blue circle represent genes, and gray diamond represent reactions

future PN. In addition, the early-treatment results suggest that paclitaxel-induced metabolic changes may be related to the disruption of energy homeostasis, in particular glycolysis and the TCA cycle. However, early metabolic changes induced by paclitaxel treatment do not appear to be informative of the severity of future PN.

Importantly, our findings have plausible neurobiological explanations. Histidine is an essential amino acid and a precursor of histamine, a neuromodulator implicated in the pathogenesis and inflammatory process of neuropathic pain [37–39]. Phenylalanine is also an essential amino acid and a precursor of phenylethylamine and dopamine, both of which are neuromodulators implicated in the pathogenesis of certain psychiatric and neurological diseases [40, 41]. Interestingly, phenylalanine is metabolically related to glutamine, which has been evaluated as a neuroprotective agent, but our findings do not suggest any association between glutamine and PN (Supplemental Table 1). Threonine, another essential amino acid, has not been shown to directly play a role in PN; however, elevated plasma threonine concentration can

lead to the accumulation of glycine in the brain which has been shown to affect neurotransmitter balance during early postnatal life [42].

Although our analysis did not show that paclitaxel-induced changes in metabolite concentrations were associated with the future development of PN, the elevation in 2- and 3-hydroxybutyrate concentrations concurrent with lower pyruvate, alanine, and threonine concentrations at EOI (Fig. 4b) are suggestive of mitochondrial dysfunction and/or oxidative stress [43]. In animal tissue studies, paclitaxel induces mitochondrial structural changes due to inflammation [44–46] and causes mitochondrial dysfunction manifested as a persistent energy deficiency [47]. Both features were related to painful PN in these studies. Collectively, the distinct metabolic pattern at EOI (Fig. 4b) and 24H (Fig. 4c) provides some evidence for a series of sequential metabolic responses important in energy homeostasis.

The clinical implications of our findings could lead to improvement in paclitaxel-induced PN prevention. Genomics- [48–50] and proteomics-based PN [51] studies have

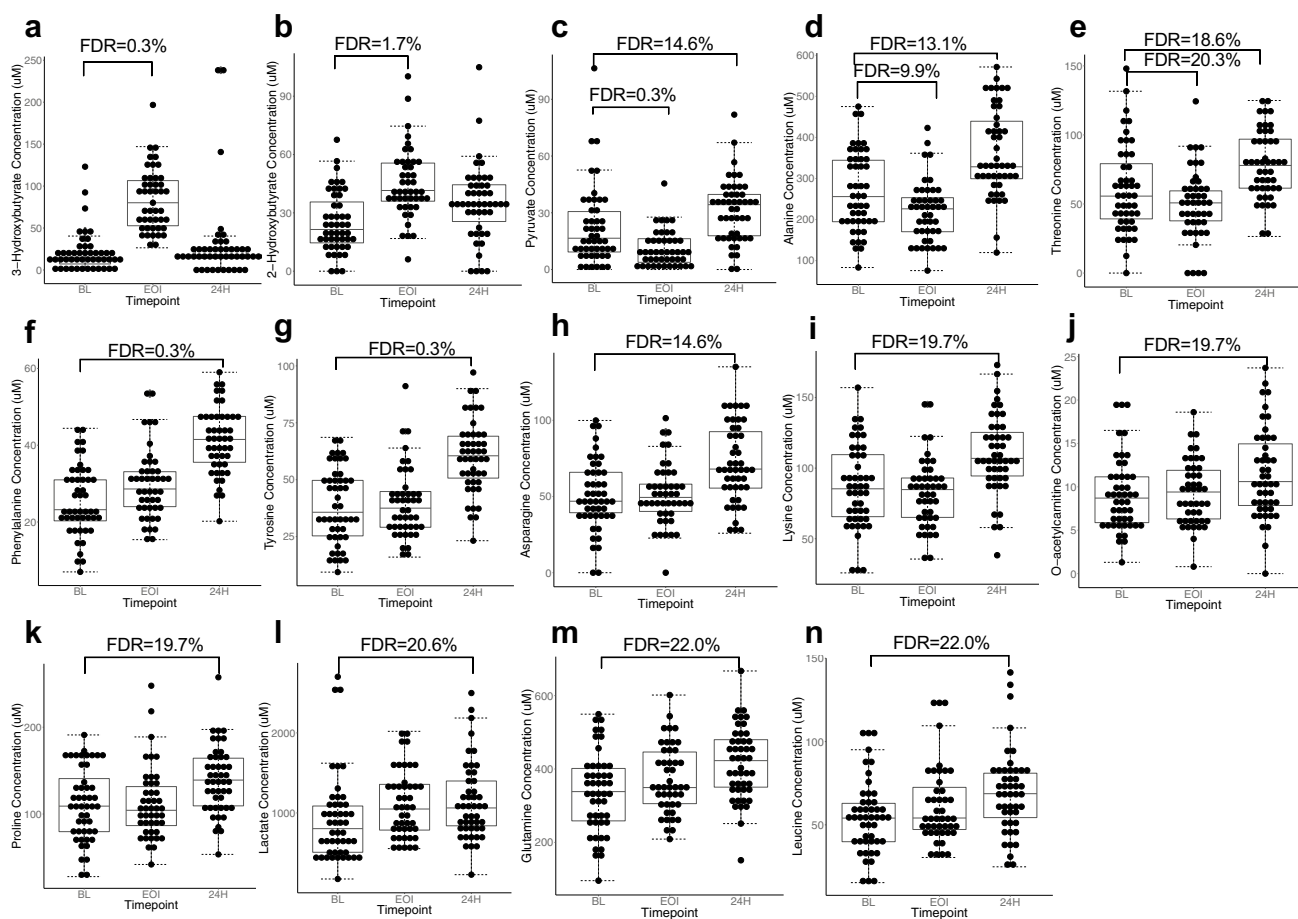


Fig. 3 Impact of the first dose of paclitaxel on individual metabolite concentrations at the end of infusion (EOI) and after 24 h (24H). The concentrations of 14 metabolites, **a** 3-hydroxybutyrate, **b** 2-hydroxybutyrate, **c** pyruvate, **d** alanine, **e** threonine, **f** phenylalanine, **g** tyrosine, **h** asparagine, **i** lysine, **j** o-acetylcarnitine, **k** proline, **l** lactate, **m** glutamine, and **n** leucine changed significantly from BL to EOI, from

BL to 24H, or both (FDR \leq 25%). Data are represented by individual concentration measurements in a box and whisker plot in which the box represents interquartile. None of the metabolic changes from BL to EOI is significantly correlated with maximum change in CIPN8 score by Pearson correlation corrected by FDR (FDR \leq 25%). KEGG IDs of metabolites can be found in Supplemental Table 3

not yielded clinically useful biomarkers, but the trend of utilizing systems biology to better understand and predict PN has gained traction. Our preliminary results support continued work in the pharmacometabolomics field that will require further testing and validation using existing blood samples from completed clinical trials [52] as well as prospective verification in future clinical trials to accurately define metabolite concentration thresholds associated with the development of PN. By identifying the subset of patients at risk for PN, more frequent PN monitoring can be implemented and paclitaxel dosing regimens can be tailored to maximize efficacy and minimize toxicity [6, 25]. In addition, the metabolite concentration thresholds can be used to identify patients susceptible to PN for enrollment in clinical studies evaluating novel techniques or agents for PN prevention and treatment.

Although our results are provocative, there are a few limitations that warrant consideration. First, this is a

feasibility study with a small sample size. We elected to use a liberal FDR of 25% to identify differentiating metabolites and capture important metabolic patterns that may be associated with the development of PN. This liberal FDR cut-off has been used in a previous proteomics study [51]. Second, NMR metabolomics only captures a small fraction of the entire metabolome. Given the inconclusive findings of previous clinical PN prevention and treatment studies about the role of certain antioxidants, vitamins, and amino acids [7–15], and the preliminary nature of our study, we selected to employ quantitative NMR platform because of its advantages of reproducibility, quick turn-around time, and its utility for WB metabolomics [53]. Lastly, dietary and supplement information was not collected from enrolled patients. The range of pretreatment histidine, phenylalanine, and threonine concentrations was broad. Since these differentiating metabolites are essential

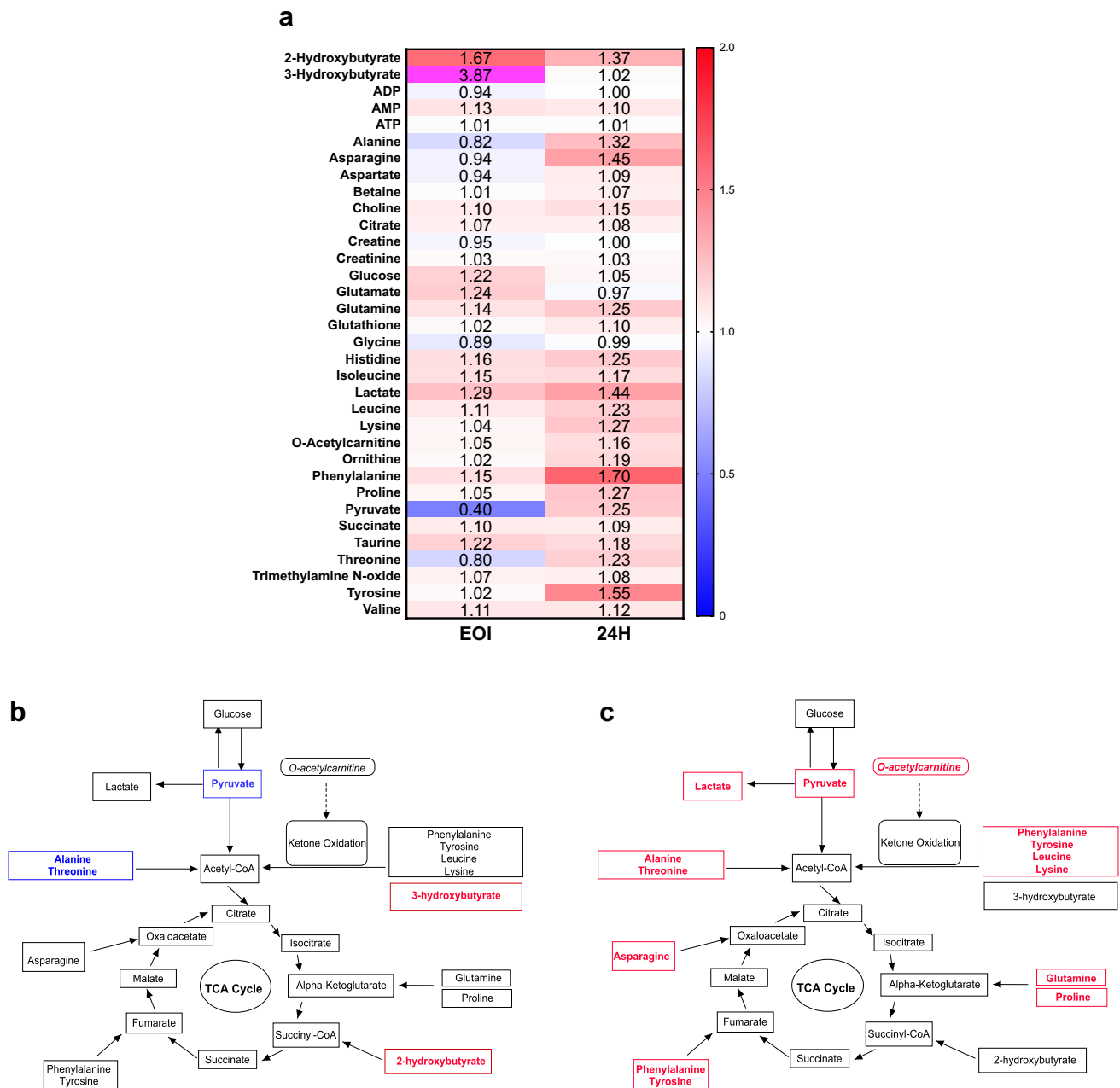


Fig. 4 First dose of paclitaxel disrupts energy homeostasis. A heatmap **a** scaled by median fold-change of each metabolite concentration from BL to EOI (first column) and from BL to 24H (second column). Red represents the increase in concentration (fold-change > 1) and blue represents the decrease in concentration (fold-change < 1) from BL. The metabolic pattern at EOI and 24H is illustrated in a biological context through MetScape mapping. Metabolites with $FDR \leq 25\%$ are uploaded into MetScape and the resulting metabolic networks

represented by these metabolites were simplified in panel **(b)** and panel **(c)**. The primary metabolic pathways disrupted by first-dose paclitaxel are glycolysis and tricarboxylic acid cycle (TCA). In both panels, the concentrations of metabolites in red are increased and those in blue are decreased. In aggregate, these findings suggest that there is a series of sequential energy metabolic responses induced by paclitaxel

amino acids, we could not rule out the possibility that differences in diet may contribute to our findings.

In conclusion, this study demonstrates the feasibility of whole blood metabolomics, together with PRO data, to discover potential clinical biomarker of paclitaxel-induced

PN. Pretreatment histidine, phenylalanine, and threonine concentrations may have predictive value for the severity of treatment-emergent PN. Confirmatory studies are needed to validate these candidate biomarkers and extend the scope of our findings by employing a more detailed metabolomics

approach [e.g., liquid chromatography (LC)–mass spectrometry (MS)] [54, 55]. We envision that a predictive model, incorporating the PN biomarkers from metabolomics validation studies, could be transformed into a decision-making algorithm that can help tailor PN monitoring and/or guide paclitaxel dosing to enhance efficacy and prevent unnecessary PN in patients with breast cancer.

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Compliance with ethical standards

Conflict of interest Dr. Daniel F. Hayes reports financial interest with Oncimmune LLC and Inbiomotion, serves as a consultant for Cepheid (no compensation), receives research grants from Merrimack Pharmaceuticals, Inc, Eli Lilly Company, Menarini/Silicon BioSystems, Puma Biotechnology, Inc, Pfizer, and Astra Zeneca, receives royalties from licensed technology from Janssen R&D, LLC, and holds three patents. The rest of the authors declare no conflict of interest.

Research involving human participants The study was approved by the University of Michigan IRBMed (HUM 00086253). All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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