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Urinary metabolite profiles associated with Multiple Chemical Sensitivity: a pilot study

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Urinary metabolite profiles associated with Multiple Chemical Sensitivity: a pilot study

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B.S.
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ABSTARCT

Urinary metabolite profiles associated with Multiple Chemical Sensitivity: a pilot study

By: Kelsey A. Fuchs

Purpose: To perform a pilot test to discover urinary metabolites associated with Multiple Chemical Sensitivity (MCS), an environmentally triggered and potentially debilitating condition.

Methods: Urine samples were collected from female participants (n=8) with or without MCS consecutively for 5 days and analyzed using ultra high-resolution liquid chromatography mass spectrometry. Data were analyzed with biostatistics, bioinformatics, and metabolic pathway enrichment. Levels of urinary metabolites were creatinine normalized. Enriched metabolites were identified based on MSI criteria.

Results: A total of 1284 metabolic features in urine samples differed between MCS cases and controls ($P<0.05$). Metabolites were enriched in immune response pathways such as histidine metabolism. There were 16 confirmed metabolite identities associated with perturbations in histidine metabolism, 9 of which differed between groups (e.g. higher urinary histamine in MCS cases; $P< 0.05$). Results indicated a potential role of altered metabolism in MCS cases.

Conclusion: This pilot study detected higher levels of inflammatory metabolites in individuals with MCS. This proof-of-concept study also establishes the feasibility of global metabolic profiling of MCS urine as a non-invasive and affordable approach to aid in mechanistic research of MCS and inform precision medicine.

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INTRODUCTION

Multiple Chemical Sensitivity (MCS) is an environmentally triggered syndrome characterized by the manifestation of adverse health outcomes upon low-level exposures to common environmental chemicals. Triggers include perfumes, insecticides, flame retardant treated carpets, petrol fumes, cigarette smoke, cleaning products, and more. Symptoms involve multiple organ systems and can include headache, dizziness, nausea, muscle and joint pain, respiratory problems, gastric problems, extreme fatigue and unusual memory loss. After exposures are removed, symptom improvement can take a few hours to several days, and sensitivity can increase over time (Rossi & Pitidis, 2018). A recent study on national prevalence of MCS found that 12.8% of adult Americans reported medically diagnosed MCS and 25.9% self-reported chemical sensitivity (Steinemann, 2018). The etiology of MCS and underlying pathological mechanisms are unknown. Consensus criteria for a case definition of MCS has not been updated since 1999 (Arch Environ Health, 1999). Therefore, understanding potential causes of MCS and the mechanisms leading to its debilitating symptoms are crucial for disease prevention and treatment.

MCS is a highly under-researched chronic condition affecting the lives of many individuals who struggle seeking therapeutic care (Gibson et al., 2016). However, a handful of dedicated researchers have been searching for reliable biomarkers of disease in MCS by testing for genetic, immunologic, and metabolic markers (De Luca et al., 2011). It is suggested that individuals may be pre-disposed to MCS through genetic variants affecting xenobiotic metabolism. MCS polymorphisms are found in cytochrome 450 enzymes (CYPs), glutathione-S-transferases (GSTs), UDP-glucoronosyl transferases (UGTs), N-acetyl transferases (NATs), paraoxonases (PONs), and superoxide dismutases (SODs) (Baines et al., 2004; Hojo et al., 2009). Others suggest

these polymorphisms reflect the general population rather than MCS specifically, and thus altered patterns of redox and cytokine activity associated with MCS may be due to inhibition of gene expression or enzyme activity (De Luca et al., 2010). A recent study found increased plasma levels of pro-inflammatory cytokines and interleukin-2 associated with MCS (Dantoft et al., 2014). As case-control studies aim to identify immunologic profiles unique to MCS, there is no consistency in altered parameters across multiple studies (Bornschein et al., 2008). Inconsistencies may arise from wide range of case definitions used and low number of epidemiologic MCS studies performed.

Regarding metabolism, studies with PET scans identified increased metabolism in the olfactory brain regions among MCS cases upon chemical exposure (Alessandrini et al., 2016). Several studies suggest neurogenic inflammation as the principal mechanism underlying MCS based on similar disease characteristics with chronic fatigue syndrome and fibromyalgia (Pall, 2003; McFadden, 1996). However, only a couple studies have been able to quantify inflammatory metabolites associated with MCS. One study looked at human MCS plasma and found increased levels of nitrites/nitrates, decreased glutathione reduced form, and decreased linolenic and arachidonic acids (De Luca et al., 2010). Mechanistic studies such as these are lacking in sheer quantity, which reflects the global issue that information on MCS etiology and pathophysiology is scarce. Many questions and few consistencies exist regarding our biological understanding of this disease. Therefore, more research is needed to explore a full spectrum of altered metabolic pathways potentially associated with MCS.

Only one study has attempted to characterize the entire metabolic profile – the metabolome – for individuals clinically diagnosed with MCS. Results showed higher levels of environmental chemicals, such as pelargonic acid herbicide, as well as lower

levels of the endogenous metabolite, acetylcarnitine, in Japanese patients with MCS (Katoh et al., 2016). Metabolomics research is a quickly expanding field based on advancements in ultrahigh resolution mass spectrometry and bioinformatic data analysis using advanced computational algorithms (Uppal et al., 2016). High-resolution metabolomics can measure metabolites of over 100 metabolic pathways and provide global information on the biochemical processes. Such techniques prove useful for environmental health research by characterizing metabolomes associated with metal exposure and adverse health outcomes (Chandler et al., 2016; Eguchi et al., 2018).

In the present study, we employ high-resolution metabolomics (HRM) as a non-targeted biomonitoring approach to discover metabolic profiles unique to MCS. This pilot study is the first of its kind to assess the metabolome of a United States-based MCS cohort. We conducted metabolomics and pathway analysis of urinary metabolites altered between study group participants (n=3 MCS cases; n=5 non-MCS controls). Our study aims to establish the feasibility of global metabolic profiling to promote further investigation into mechanisms underlying MCS. Additionally, urinary analysis serves as a non-invasive and low-burden method for assessing MCS metabolism, as compared to collecting blood plasma, because it is more affordable, non-invasive, and minimizes symptom-triggering exposures. This study brings the MCS community under the scope of advanced biomedical research to expand our understanding of the disorder and inform precision medicine.

METHODS

Study Population and Urine Collection

The study subjects consisted of 4 MCS (case) and 5 non-MCS (control) female participants residing in the Atlanta area (GA, USA). Cases were defined as having MCS

for at least 5 years, while controls were healthy women without history of allergies, sensitivity to chemicals or food items, respiratory or other chronic diseases, and use of any chronic medications during the previous 12 months. Ages of selected participants ranged from 48-80 for MCS and 24-60 for non-MCS. One MCS case dropped out and the remaining 8 participants completed 5 consecutive days of follow-up in July of 2018. Each day participants collected their bedtime urine (last void prior to sleep), any nighttime voids, and their first morning void in separate labeled containers. Specimens were stored in participants' refrigerators until transported to the laboratory for analysis. Analysis of urine collected from bedtime to first morning voids were considered a proxy for the previous day's exposures. All participants provided informed consent and the study was approved by the Emory University Institutional Review Board.

Sample Preparation for High-Resolution Mass Spectrometry

For metabolomics analysis, 100 µl of acetonitrile containing internal standards were added to 50 µl of urine samples and allowed for equilibration on ice for 30 min, then centrifuged (16.1 x g at 4dC) for 10 min to remove precipitates. Supernatants were added to autosampler vials and stored at -80 ºC until analysis (Chandler et al., 2016).

High-Resolution Mass Spectrometry and Data Extraction

Prepared samples were injected and analyzed using liquid chromatography (LC) with Fourier transform mass spectrometry (Dionex Ultimate 3000, Q-Exactive HF, Thermo Scientific). For 60 urine samples (15 from 3 cases, 45 from 5 controls), 10 µL aliquots of each sample were analyzed in triplicate using hydrophilic interaction liquid chromatography (HILIC) with electrospray ionization (ESI) source operated in positive mode and C18 hydrophobic reversed-phase chromatography with ESI operated in negative mode (Walker et al., 2019). Analyte separation for HILIC was performed with a

Waters XBridge BEH Amide XP HILIC column (2.1 mm x 50 mm, 2.6 µm particle size); C18 chromatography was performed on an end-capped C18 column (Higgins Targa C18 2.1 mm x 50 mm, 3 µm particle size). The high-resolution mass spectrometer operated at 120,000 resolution while scanning a mass-to-charge ratio (*m/z*) range of 85-1275. Two quality control pooled reference plasma samples, NIST 1950 (NIST, 2014) and QStd-3, were included at the beginning and end of each batch of 20 samples for normalization, batch effect evaluation, and post hoc quantification. Raw data files were extracted and aligned using apLCMS (Yu et al., 2013) followed by xMSanalyzer (Uppal et al., 2013). Detected ions, referred to as *m/z* features, consisted of their unique mass-to-charge ratio (*m/z*), retention time, and ion abundance. Prior to data analysis, batch-effect correction was performed by ComBat (Johnson et al., 2007).

Creatinine Levels Quantitation

Levels of creatinine in individual urine samples were quantified based on colorimetric detection using a creatinine assay kit (ab65340, Abcam). Ion abundance of *m/z* features were normalized to individual sample creatinine levels to account for differences in water consumption and urine dilution across days and between study participants.

Metabolomics Data Analysis

Intensities for each *m/z* feature were summarized using the median of three replicates. Data was then filtered to only keep features with at least 80% non-missing values across all samples and 80% non-missing values in at least one group. Data was further processed by log2 transformation, then quantile normalization. A two-way ANOVA repeated measures using LIMMA was performed to find *m/z* features that significantly differed between MCS cases and controls. Metabolites with raw $P < 0.05$ were selected for metabolic pathway analysis. Using FDR adjustment was not realistic due to the small

number of subjects. Therefore, we used a less stringent criteria to obtain optimal coverage of metabolites (100-500 metabolites) for permutation testing. In addition to the limma test, pathway enrichment analysis protects against type 1 statistical error. Final data was visualized with unserpervised two-way hierarchal clustering analysis (HCA) to illustrate the clustering pattern of selected *m/z* features and samples by group. Levels of selected metabolites were quantified by using reference standardization with NIST 1950 certified values (Go et al., 2015).

Pathway Analysis

To define metabolic pathways associated with changes in metabolism between groups, *mummichog* pathway analysis was performed (Li et al., 2013). Selected *m/z* features with corresponding P values from positive and negative mode ionizations were inputted into *mummichog2.0* to search for (M+H) and (M+Na) adduct matches, and (M-H), (M-H-H), (M-H2O), (M+Cl) adducts, respectively. The algorithm performed 100 permutation tests with $P < 0.05$ to identify significant modules and metabolic pathways that are most likely to be associated with true biological activity networks. Significant pathways were then cross-referenced in Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.genome.jp/kegg/pathway.html) to confirm directionality of mapped metabolites.

Metabolite Annotation and Identification:

Metabolites were annotated by matching accurate mass *m/z* for adducts formed under positive and negative ESI conditions using xMSannotator (Uppal et al., 2017), which cross-references physiochemical properties of possible chemical identities with online databases such as Human Metabolome Database (HMDB) (Wishart et al., 2012) and KEGG, with a match tolerance of 10 ppm.

Levels of identification confidence were assigned using Metabolomics Standards Initiative (MSI) (Sumner et al., 2007). Identities of selected metabolites were confirmed by co-elution and ion dissociation mass spectrometry (MS/ MS) relative to an internal library of 500 authentic reference standards analyzed under identical experimental conditions (Liu et al., manuscript submitted) [Level 1 identification]. In the absence of authentic standards, putative identities were confirmed by MS/ MS relative to spectral databases such as METLIN (http://metlin.scripps.edu) or MetFrag (Ruttkies et al., 2016) [Level 2 (high) identification]. Level 2 (medium) was assigned to metabolites whose identify was matched to multiple co-eluting adducts in addition to its most common adduct, M+H adduct detected in positive mode or M-H detected in negative mode. Having just the M+H or M-H adduct as well as multiple metabolites from the same pathway received Level 2 (low) identification. Level 3 identification was conservatively assigned to tentative candidates of compound classes (Wang et al., 2018).

RESULTS

High-Resolution Metabolomics

Urine samples were collected from individuals suffering from Multiple Chemical Sensitivity to investigate possible alterations in their daily metabolism upon exposure to low levels of ubiquitous, man-made chemicals. Analysis was conducted with an established high-resolution metabolomics workflow (Walker et al., 2019). Extraction and processing of the LC-MS data resulted in two feature tables, one from ionization on the HILIC+ column, and one from the C18- column. Each feature table contained unique *m/z* features detected among 40 urine samples from n=8 over 5 days. Of the 16068 *m/z* features detected via HILIC+, 984 metabolic features differed significantly between MCS cases and controls (raw $P < 0.05$). Of the 6547 *m/z* features detected via C18-, 300 metabolic features differed significantly between MCS cases and controls (raw $P < 0.05$).

Unsupervised hierarchical clustering of these significant features was visualized for both ionization modes in Figure 1A-B.

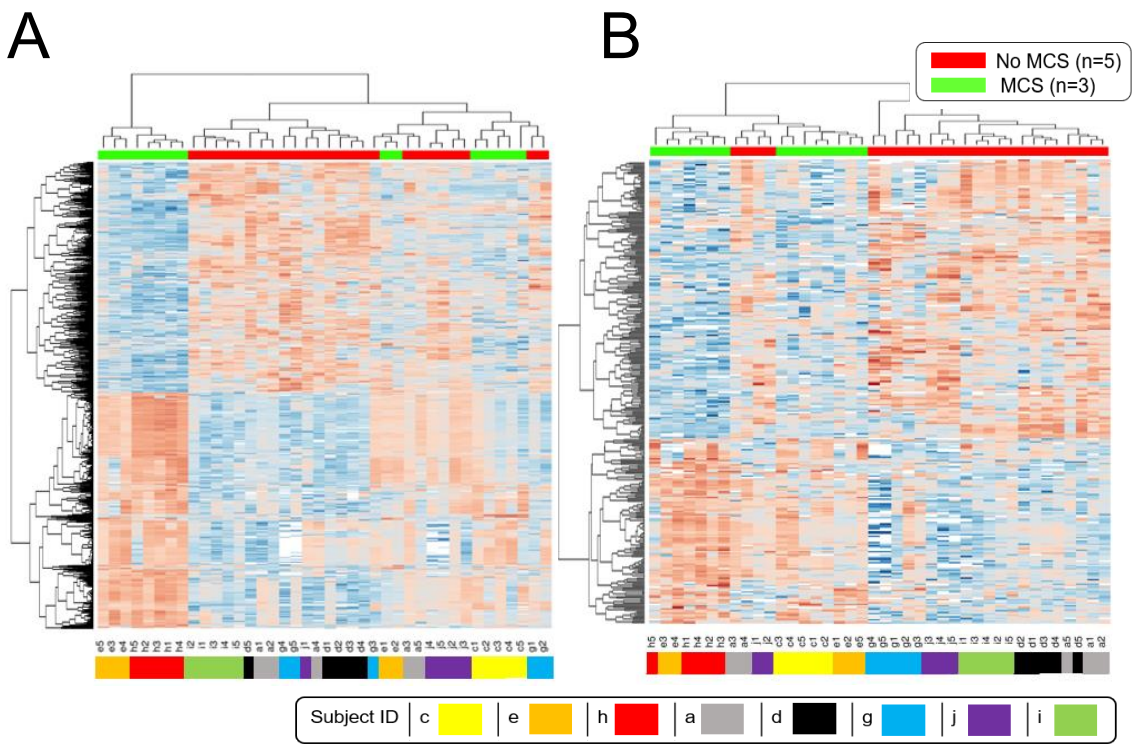

Figure 1. Unsupervised two-way hierarchical cluster analysis (HCA) and heat maps showed differences in metabolic response between MCS and non-MCS (control) urine. Clustering of 40 samples (n=8, 5 days) with relative abundance of significant *m/z* features (raw $P < 0.05$). **(A)**. Data were collected on HILIC column with positive ionization mode. Displaying 984 *m/z* features. **(B)**. Data were collected on C18 column with negative ionization mode. Displaying 300 *m/z* features.

In Figure 1A-B, the MCS samples (green) clustered as the control (red) samples, indicating more similarities within groups than between groups. Moreover, rows represent individual *m/z* features and their relative abundance in each sample shown by a z-score of 4 to -4. For example, the furthest left cluster of 8 MCS samples (e & h) in

Figure 1A aligned based on similar ion abundances across most features. This specific cluster of MCS samples had 498 metabolic features in greater abundance (dark red) in their urine compared to non-MCS urine, and 486 metabolic features in less relative abundance (dark blue) as detected with HILIC+. Overall, results from unsupervised two-way HCA of both detection modes show daily urine samples of one study participant clustering together more often than with another participant's samples (color-coded by the subject ID, Figure 1A-B). These results indicate MCS subjects have different metabolic profiles compared to healthy controls, and thus should be investigated further.

Pathway Enrichment Analysis

We used a non-targeted approach to identify biological pathways associated with metabolic differences among MCS cases. This top-down profiling method successfully detects metabolites in targeted pathways and metabolites in unsuspected pathways to best capture disease-associated metabolomes (Johnson et al., 2010). Pathway enrichment analysis tested for whether a list of metabolic features was altered more than would be expected by chance (Reimand et al., 2019). Algorithms used, *mummichog2.0,* were able to predict functional activity from our spectral feature tables based on collective power of known metabolic pathways and networks, without a priori identification of metabolites (Li et al., 2013).

With positive ionization feature table, we identified 984 *m/z* features with corresponding raw P values. These 984 features showed enrichment across ten significant pathways, as shown in Figure 2A. Top three pathways were prostaglandin formation from arachidonate, leukotriene metabolism, and histidine metabolism, all of which contain several inflammatory metabolites and play key roles in immune response. Algorithms matched the 984 significant *m/z* features to 150 Empirical Compounds from a reference

network of 838 Empirical Compounds. An Empirical Compound is a computational unit for a tentative metabolite; it groups multiple potential ions for potential adducts of the tentative metabolite (Li et al., 2013). Of all ten pathways listed in Figure 2A, histidine metabolism had greatest overlap size of 11/33, meaning a total of 33 Empirical Compounds (i.e. tentative metabolites) matched to our feature table were related to histidine metabolism, 11 of which showed a statistically significant association with said pathway via permutation testing. Therefore, histidine metabolism was selected for further investigation of key metabolites.

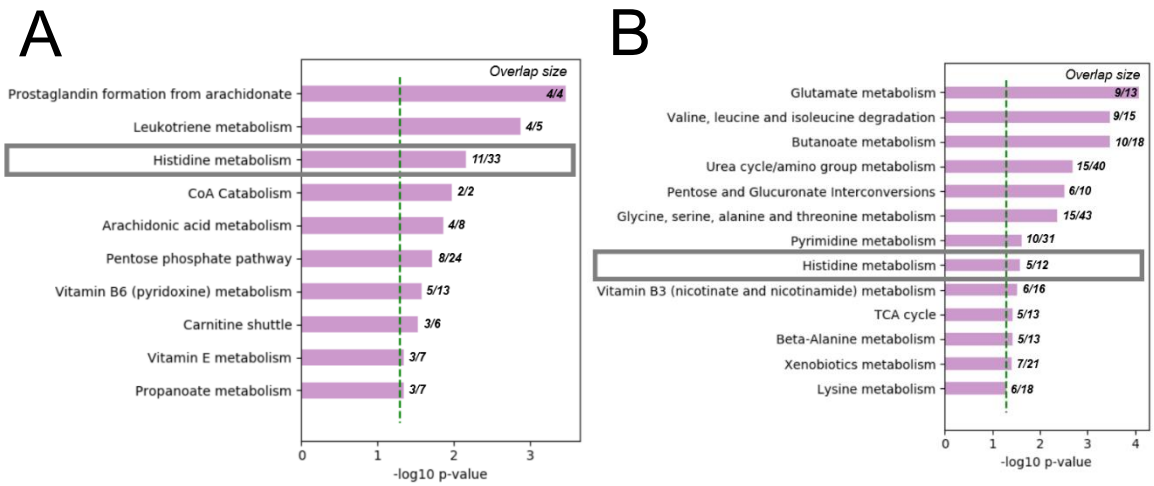

Figure 2. Pathway enrichment analysis of MCS urinary metabolites. Pathway enrichment rate analysis of MCS urine compared with non-MCS control urine. **(A)**. A total of (10/ 119) enriched pathways determined significant ($P < 0.05$; as shown by dotted line), using HILIC+ feature table. **(B)**. A total of (13/ 119) enriched pathways determined significant ($P < 0.05$), using C18- feature table.

With negative ionization (Figure 2B), 300 *m/z* features were detected with raw P values < 0.05. Overall, algorithms matched our inputted feature table to 99 Empirical Compounds from a reference map of 514. Glutamate metabolism is the top pathway in the list of 13 significant pathways shown in Figure 2B. Glutamate acts as a precursor or

substrate in the biosynthesis of several amino acids, nucleic acids, nucleotides and metabolites (Yelamanchi et al., 2016). In fact, 10 of the 13 significantly enriched pathways listed in Figure 2B contained glutamate, including glutamate metabolism and histidine metabolism. Histidine metabolism was the only significantly enriched pathway detected in both positive and negative ionization modes. Therefore, histidine metabolism was selected for further investigation of key metabolites.

Histidine Metabolism

Our targeted focus on histidine metabolism resulted from non-targeted pathway analysis. A total of 16 *m/z* features significantly enriched histidine metabolism. Tentative metabolites were selected when common adducts, M+H or M-H, were present. Tentative identities represented Level 3 identification confidence according to Metabolomics Standards Initiative (MSI) criteria. Metabolite identification shifted to Level 2 confidence when adducts were in agreement with annotations by xMSannotator (Uppal et al., 2017). Putative identities from xMSannotator were compared to authentic chemical standards. Level 1 confidence was assigned when accurate mass and retention time of detected features were comparable. Overall, we identified 16 metabolites associated with histidine metabolism. Table 1 lists each metabolite characterized by accurate mass-to-charge ratios (*m/z*), retention times for how long the ions remained on the liquid chromatography columns, adduct(s) formed upon ionization, and confidence levels of identification.

Table 1. Identification of selected metabolic features for histidine metabolism.

Name	m/z	Retention Time (s)	Adduct	Confidence Level
Glutamate	149.0638	98.9	M+H	1
Glutamate	146.0460	34.9	M-H	1
Glutamate	128.0353	35.1	M-H2O-H	3
4-Imidazolone-5-propanoate	155.0463	43.9	M-H	2 (low)
4-Imidazolone-5-propanoate	179.0427	94.9	M+Na	2 (medium)
4-Imidazolone-5-propanoate	157.0608	95.9	M+H	2 (medium)
Formiminoglutamate	173.0568	33.7	M-H	2 (high)
Formiminoglutamate	175.0714	91.2	M+H	2 (high)
2-Hydroxyglutarate	147.0300	36.8	M-H	2 (high)
2-Hydroxyglutarate	149.0431	46.3	M+H	2 (low)
2-Oxoglutarate	145.0143	34.5	M-H	2 (high)
Glutarate	131.035	42.0	M-H	1
Fumarate	115.0037	37.3	M-H	1
β-alanine	88.0404	41.6	M-H	1
Carnosine	227.1135	133.8	M+H	1
Carnosine	226.1067	58.2	M[1+]	3
Histamine	112.0869	130.6	M+H	1
Methylhistamine	126.1026	118.3	M+H	1
Histidine	154.0623	45.1	M-H	1
Methylhistidine	168.0779	46.3	M-H	1
Imidazole-4-acetaldehyde	111.0553	103.0	M+H	2 (low)
Imidazole-4-acetate	125.0356	43.8	M-H	1
Imidazole-4-acetate	127.0502	81.7	M+H	1
Methylimidazole acetaldehyde	125.0709	131.9	M+H	2 (low)

Ten metabolites were identified with Level 1 confidence (Table 1). Common adducts for Level 1 metabolites were graphically represented in Figure 3. Median ion abundances for 8 histidine-related metabolites were higher among MCS cases than that of controls (histamine, histidine, glutamate, fumarate, methylhistamine, carnosine,

formiminoglutamate, imidazole-4-acetate) (Figure 3). However, the overall distributions of methylhistamine and fumarate were comparable between MCS cases and controls (Figure 3). Histamine levels were statistically significantly higher in MCS urine relative to control (*P < 0.05 student's t-test).* Histamine acts as neurotransmitter in the hypothalamus to regulate circadian rhythms (Hsieh et al., 2019). Histamine secreted from mast cells plays key roles in allergic response and inflammation from tissue damage (Pagliarini et al., 2016).

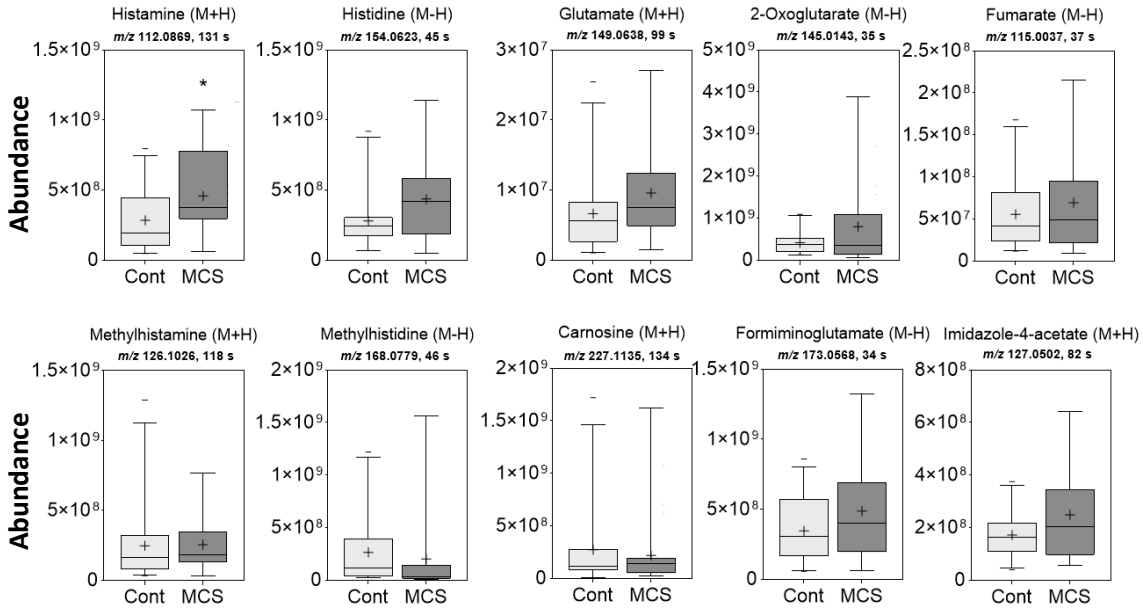

Figure 3. Creatinine-normalized abundance of selected metabolites differ between MCS and non-MCS (control) urine. **P < 0.05 student's t-test.*

Figure 4 shows the connectedness of all 16 key metabolites involved in histidine metabolism pathway. To avoid making skewed comparisons of overall ion abundance between cases and controls, both the mean and the median ion abundance for MCS cases were entirely above or below corresponding non-MCS control parameters in order to be shaded red or blue, respectively (Figure 4).

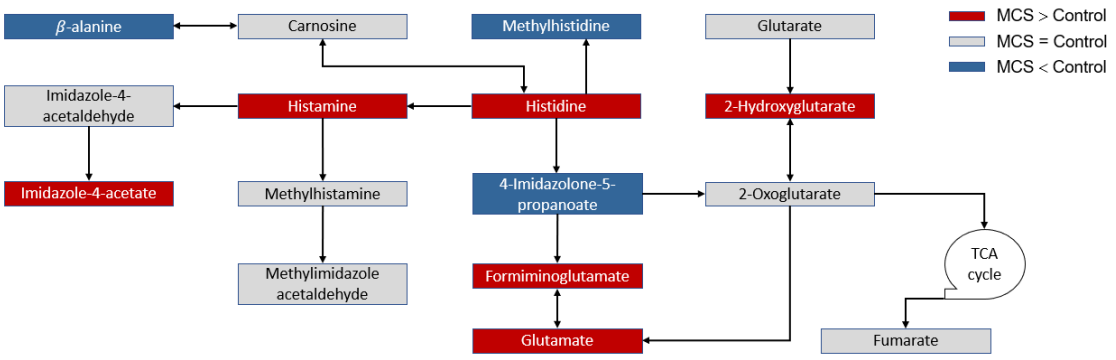

Figure 4. Mapped pathway of selected metabolic features for histidine metabolism.

Including histidine, the central molecule of this pathway, 6 identified metabolites were detected in overall greater abundance for MCS cases relative to their non-MCS counterparts (Figure 4 *red*). Abundances of 7 intermediate metabolites were distributed equally across both groups (Figure 4 *gray*). We detected lower abundance of 3 metabolites among MCS cases versus controls (Figure 4 *blue*).

DISCUSSION

Overall, results from HRM of MCS urine showed metabolic differences among MCS cases, and one perturbed central pathway was histidine metabolism. Higher abundance of several inflammatory markers, such as histamine, were found in MCS urine samples. Metabolomics data such as this can be used to test associations with specific exposure, health behavior, and demographic characteristics of study participants in metabolome-wide association studies (MWAS) to understand the risk factors for the metabolic perturbations. For example, a recent study plasma levels of carnosine and methylhistamine among healthy individuals detected through LC-MS were positively associated with meat consumption, especially for processed meats (Mitry et al., 2019). Another study showed that inhibition and overexpression of the transcription factor,

MYC, disrupted normal circadian rhythms and histidine and histamine levels (Hsieh et al., 2019). Interestingly, histidine can only be converted to glutamate in the liver, while conversion to histamine can occur anywhere, even though to a much lesser extent (Pagliarini et al., 2016). On the other hand, previous studies have suggested that xenobiotic metabolism may be altered among MCS, and majority of this metabolism would take place in the liver (Baines et al., 2004; Hojo et al., 2009).Therefore, liver metabolism of histidine may play an important role in MCS and studies can also use HRM results to investigate gene-environment interactions affecting liver metabolism.

The regulatory effects of histamine among MCS should continue to be investigated. Researchers measured higher levels of histamine in MCS serum as compared to controls (De Luca et al., 2010). Since the olfactory regions of the brain are near the histaminergic neurons of the limbic system, models of neuronal hypersensitivity in these areas have also been proposed as mechanisms of MCS (Alessandrini et al., 2016). Thus, exposure assessments involving olfactory stimulation among MCS could incorporate HRM data as potential markers of altered neuronal response. Data from this study are not enough to make significant claims on certain metabolites, such as histamine, acting as reliable biomarkers for research because of our small sample size (n=8) and lack of statistical power. However, our pilot study has demonstrated the utility of urinary metabolomics in MCS research and the biological relevance to metabolism perturbation. More studies with a larger sample size and longer longitudinal follow-up are warrantied and will address day-to-day variability of urinary metabolites.

Our goal was to explore urinary metabolomics as a non-invasive and affordable approach for assessing MCS-associated metabolism. This goal was encouraged by the strong need for more biomedical research regarding MCS while causing little to no harm

to MCS cases during assessment. Urine sample collection minimized participants' exposure to clinicians and medical equipment needed for blood sampling, which also reduced cost. As this study was the first of its kind, results showed that innovative HRM methodology was capable of characterizing metabolic profiles of human participants. This proof-of-concept study establishes the feasibility of global metabolic profiling to promote future research into precision medicine and further investigation into mechanisms underlying MCS. We hope to build a research program providing a practical approach integrating robust biomedical research with community needs. Such research will lead to understanding of biological mechanisms underlying chemical sensitivity and evaluation of interventions to inform precision medicine.

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