Journal of Neuroimmune Pharmacology (2019) 14:241–250
https://doi.org/10.1007/s11481-018-9815-4

ORIGINAL ARTICLE

Urinary and Plasma Metabolomics Identify the Distinct Metabolic Profile of Disease State in Chronic Mouse Model of Multiple Sclerosis

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Received: 11 July 2018 / Accepted: 5 October 2018 / Published online: 12 October 2018

Abstract

Identification of non-invasive biomarkers of disease progression in multiple sclerosis (MS) is critically needed for monitoring the disease progression and for effective therapeutic interventions. Urine is an attractive source for non-invasive biomarkers because it is easily obtained in the clinic. In search of a urine metabolite signature of progression in chronic experimental autoimmune encephalomyelitis (EAE), we profiled urine at the chronic stage of the disease (day 45 post immunization) by global untargeted metabolomics. Using a combination of high-throughput liquid-and-gas chromatography with mass spectrometry, we found 105 metabolites ($P < 0.05$) significantly altered at the chronic stage, indicating a robust alteration in the urine metabolite profile during disease. Assessment of altered metabolites against the Kyoto Encyclopedia of Genes and Genomes revealed distinct non-overlapping metabolic pathways and revealed phenylalanine-tyrosine and associated metabolism being the most impacted. Combined with previously performed plasma profiling, eight common metabolites were significantly altered in both of the biofluids. Metaboanalyst analysis of these common metabolites revealed that phenylalanine metabolism and Valine, leucine, and isoleucine biosynthetic pathways are central metabolic pathways in both bio-fluids and could be analyzed further, either for the discovery of therapeutics or biomarker development. Overall, our study suggests that urine and plasma metabolomics may contribute to the identification of a distinct metabolic fingerprint of EAE disease discriminating from the healthy control which may aid in the development of an objective non-invasive monitoring method for progressive autoimmune diseases like MS.

Keywords Metabolomics · EAE · Multiple sclerosis · Metabolomics · Urine · Biomarkers · GC-MS/LC-MS · Metabolic pathways

Introduction

Multiple sclerosis (MS) is an autoimmune disease characterized by inflammatory demyelination, axonal damage and immune cell infiltration of the central nervous system (CNS). Although discovered more than a century ago, the precise mechanism of disease manifestation and the disease etiology remains elusive. The current understanding of the inflammatory aspect of MS pathogenesis has been largely complemented by research on the murine model of MS; experimental autoimmune encephalomyelitis (EAE) (Lassmann 2007; Mix et al. 2010). EAE is an antigen-driven inflammatory autoimmune model, in which immunization against myelin autoantigens elicits strong autoreactive T cell responses, leading to CNS myelin destruction (Lassmann 2007; Mix et al. 2010). EAE is a complex condition in which the interaction between a variety of immunopathological and neuropathological mechanisms leads to

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s11481-018-9815-4) contains supplementary material, which is available to authorized users.

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an approximation of the key pathological features of MS: inflammation, demyelination, axonal loss, and gliosis. SJL mice exhibit a relapsing-remitting (RR) course of EAE, whereas C57BL/6 (B6) mice display a more chronic course without complete remissions (Nath et al. 2009). The counter-regulatory mechanisms of resolution of inflammation and remyelination also occur in EAE, similar to MS, therefore making it a suitable model for the understanding of inflammation and remyelination processes.

With the rapid development of analytical techniques and advanced multivariate and bioinformatics tools, metabolomics has become a promising approach for understanding and elucidating the etiology and mechanism of disease (Wood 2014). The growing research field of metabolomics has introduced new insights into the pathology of MS/EAE (Gebregiworgis et al. 2013; Mangalam et al. 2013). Our previous work showed global plasma metabolite alteration in relapsing-remitting (SJL) and chronic (B6) mouse models of MS using untargeted metabolomics (Mangalam et al. 2013; Poisson et al. 2015b). The technique of metabolomics, in which the entire suite of metabolites produced by the body is examined, is ideally suited to the study of EAE/MS due to the severe and prevalent metabolic derangements that occur in this disease (Steen et al. 1985; Bruhn et al. 1992; Lutz et al. 2007a; Reinke et al. 2014). Metabolic alterations are also reported in MS patient-derived samples (Steen et al. 1985; Bruhn et al. 1992; Lutz et al. 2007a; Reinke et al. 2014). Isoprenoid biosynthesis was decreased in the serum from MS patients (Steen et al. 1985), and CSF metabolites from patients with active vs. inactive plaques provided a distinct metabolic profile (Lutz et al. 2007b). Two major platforms used for metabolite analysis include nuclear magnetic resonance (^{1}HNMR) and mass spectrometry (GC-MS/LC-MS) (Dunn et al. 2011). ^{1}H NMR has a dynamic range that provides broad coverage of the metabolome in a single experiment (Dunn et al. 2011). The sensitivity of GC-MS, on the other hand, facilitates identification of metabolites, some of which are not detected by NMR, and provides additional insights into the regulation of metabolic pathways (Dunn et al. 2011). A urine metabolite profile in chronic EAE mice at the peak stage of the disease and in MS patient urine samples was recently reported based on the nuclear magnetic resonance approach (^{1}H NMR)(Gebregiworgis et al. 2013).

In this study, we applied an untargeted metabolomics approach using GC-MS and LC-MS to profile the metabolic alterations in the urine of chronic EAE in B6 mice. In addition, we identified eight common metabolites in urine and plasma as potential biomarkers, which may be of clinical relevance. Specifically, these disrupted metabolic pathways may help to elucidate the pathophysiology of EAE/MS disease and facilitate the identification of novel drug targets.

Material and Methods

Animals

Female 6- to 8-week-old C57BL/6 mice were obtained from Jackson Laboratories (Bar Harbor, Maine) and were housed in pathogen-free conditions. Mice were maintained on a 12: 12-h light-dark cycle and received standard rodent chow and water ad libitum. All animal protocols were approved by the Institutional Animal Care and Use Committee of the Henry Ford Health System, Detroit, MI. EAE mice over stage 3 were afforded facile access to food and water.

Peptide and Reagents

Myelin oligodendrocytes protein peptide (MOG_{35-55}) (MEVGWYRSPFSRVVHLYRNGK) was synthesized at New England Peptide, Inc. (Gardner, MA). Complete Freund's adjuvant (CFA) and Mycobacterium tuberculosis (MT) lyophilized powder were purchased from DIFCO Laboratories (Michigan, USA).

Induction of EAE in Mice and Urine Collection

B6 mice (10-12 week old, $n = 10$) were immunized on day 0 by subcutaneous injections in the flank region with a total 200 μl of emulsion containing MOG_{35-55} peptide (300 μg/mouse), along with killed *Mycobacterium tuberculosis* H37Ra (400 μg) as described before (Poisson et al. 2015b). B6 mice were given pertussis toxin (300 ng/mouse) in the volume of 200 μl in PBS intraperitoneally on day 0 and 2 post immunization. One set of mice (n = 10) were injected with complete Freund's adjuvant (CFA)/PT without peptide named as CFA/PT control. Clinical disease was monitored daily in a blinded fashion by measuring paralysis according to the conventional grading system: 0, no disease; 1, complete loss of tail tonicity; 2, partial hind limb paralysis (uneven gate of hind limb); 3, complete hind limb paralysis; 4, complete hind and forelimb paralysis; and 5, moribund or dead. At the chronic stage of the EAE (day 45 post immunization), urine samples were collected from both CFA/PT control and EAE groups. Samples were snap frozen and stored at -80 °C.

Sample Processing for Untargeted Metabolomics

Urine samples were collected from two groups: 1) CFA/PT control, and 2) MOG-induced EAE at 45-day chronic stage ($n = 5$ / group). Samples were snap frozen till further analysis. Urine samples from two groups were analyzed individually by the Metabolon Inc. At the time of analysis, samples were extracted using Metabolon's proprietary series of organic and aqueous extractions, which are used to remove the protein fraction while allowing maximum recovery of small

molecules. The extracted samples were split into equal parts for analysis on the GC/MS (Thermo-Finnigan Trace DSQ fast-scanning single-quadrupole mass spectrometer) and LC/MS/MS (Waters ACQUITY UPLC and a Thermo-Finnigan LTQ mass spectrometer) platforms. The LC/MS portion was analyzed in two aliquots using separate acidic positive ion optimized conditions and negative ion optimized conditions. For quality assurance, several technical replicate samples created from a homogeneous pool containing a small amount of all study samples ("Client Matrix") were run. Further, QC compounds were added to each sample. Peaks were identified from the raw mass spec data files using Metabolon's proprietary peak integration software. Compounds were identified by comparison to library entries of purified standards or recurrent unknown entities. Further details of the untargeted metabolomics data generation can be found in previous publications (Mangalam et al. 2013; Poisson et al. 2015a, b).

Statistical Analysis

Metabolite intensities were obtained from Metabolon. Statistical analyses are performed with R software (http://cran.r-project.org/) and using Metaboloanalyst (http://www.Metaboloanalyst.ca/). Missing intensity data was assumed to be due to low quantities of the metabolite within the sample and were imputed using the minimum observed intensity in the study. To account for differences in urine concentration, each sample was standardized to its osmolality. The first two components of a principal components analysis (PCA) were plotted to identify whether samples or batch effects have contributed disproportionally to the variance in the data. To visualize the level of alteration in EAE urine, each metabolite was standardized to the distribution of that metabolite within CFA/PT control mice; specifically, $z_{ij} = (\text{intensity}_{ij}\text{-mean}_i)/\text{sd}_i$ for metabolite i and specimen j, where mean_i and sd_i are calculated from the CFA/PT control mouse specimens. These z-scores were plotted per metabolite (vertical axis), where color indicates diagnosis and each point represents an observation. Per-metabolite comparisons were made using two-sample t-tests. Significant differences were determined at $p < 0.05$. False discovery rates were calculated by the Q value method from the Bioconductor R package and are provided for reference. However, we use concerted pathway-level changes to select important biological contributors from among the statistically significantly altered metabolites. Heatmaps are drawn for significantly differential metabolites using blue (low) to yellow (high) coloring to depict standardized intensity differences from the metabolite-level mean. Hierarchical clustering on Pearson's correlation coefficient is used to generate all dendrograms shown. Metabolites were mapped into the murine KEGG pathways (http://www.genome.jp/kegg) using HMDB IDs and KEGG compound IDs. Pathway enrichment was assessed by a hypergeometric test of altered metabolites relative to the KEGG metabolite list. The impact of altered metabolites on the KEGG pathway topology was assessed using two well-established node centrality measures to estimate node importance - degree centrality and betweenness centrality. Degree centrality is defined as the number of links occurring upon a node. For a directed graph, there are two types of degree: in-degree for links that come from other nodes and out-degree for links initiated from the current node. Metabolic networks are directed graphs. Here we only considered the out-degree for node importance measure. It is assumed that nodes upstream will have regulatory roles for the downstream nodes, not vice versa. The betweenness centrality measures a number of shortest paths going through the node. Since the metabolic network is directed, we use relative betweenness centrality for metabolite importance measure. The degree centrality measures focus more on local connectivity, while the betweenness centrality measures focus more on global network topology (Aittokallio and Schwikowski 2006). Plots of enrichment p value (−log10, vertical axis) and impact score (horizontal axis) nominate pathways influenced by changes in EAE urine were constructed. Metabolites were mapped into the Ingenuity Pathway Analysis (IPA) software using HMDB IDs and KEGG knowledgebase of compound IDs, and networks of altered metabolites were constructed using Fisher exact test. Using R statistical computing platform method "cor", Pearson correlation coefficients, we examined the correlation between overlap metabolite intensities in plasma and in urine.

Results and Discussion

Clinical Pathology in the Chronic Model of EAE

Mice immunized with MOG_{35-55} presented with 100% disease incidence and typical EAE disease symptoms of increased clinical scores compared to CFA/PT control treated animals as before (Poisson et al. 2015b). EAE in B6 mice displayed a chronic-progressive clinical course. Mice developed the first clinical sign of EAE (limp tail) on the 15th day after MOG_{35-55} immunization and reached the peak clinical score of 3.15 ± 0.6 on the 24th day. Disease severity persisted until the end of the study (day 45; 2.8 ± 0.16) (Poisson et al. 2015b). CFA/PT control mice did not show any clinical symptoms. Urine samples were collected at the effector/chronic phase of disease (day 45 post immunization) from EAE disease and CFA/PT control mice and snap frozen for metabolomics analysis. We previously have reported that at day 45, histological analysis of spinal cords showed an extensive demyelination and antigen specific response in EAE group, whereas CFA/PT control group did not show any sign of demyelination and antigen specific response (Poisson et al. 2015b).

Urinary Metabolomics Profiles

GC-MS and LC-MS analysis were performed to determine the relative levels of metabolites across vehicle CFA/PT-control and EAE mouse urine samples. The samples had 321 metabolites detected, with minimal missing data (0.31-0.62% per sample). The missing values were imputed by half of the observed minimum value after osmolality standardization, under the assumption that missingness represents an undetectable metabolite level. Samples were then standardized as the log2 ratio relative to the osmolality constant per sample. For an overview of the GC-MS/LC-MS spectra of urine samples, a PCA of the osmolality-standardized metabolites was performed. The first two components were plotted (not shown) to depict the primary variability between the samples and to nominate potential outlying samples. Neither visible batch effects nor outlying samples were identified, so analysis proceeded with all ten samples using the imputed and osmolality-standardized data.

Urinary Profile Alteration at Chronic Stage of the EAE

Principal Components Analysis (PCA) was used to determine the ability to differentiate metabolic profiles between CFA/PT control (con) and chronic EAE groups. PCA showed clear differences in the urine metabolome between CFA/PT control and EAE group, with the data points clustered into two distinct groups (Fig. 1a), neatly separating the control and EAE samples. Though the variability is dominated by within group differences (PC1), the PC2 separation suggests that the separation of CFA/PT control and EAE urine samples can be achieved based on their metabolite make-up. By the two-sample t-test, 105 significantly altered ($p < 0.05$) urinary metabolites were identified with nine increased, and 96 decreased metabolites in the EAE group compared to controls (Supplementary Table 1). The relative profile of various urinary metabolites in EAE (red) with respect to CFA/PT controls is presented using a Z-score plot (Fig. 1b). Heat-map visualization shows the distinct segregation of control metabolites from that of EAE (Fig. 1c). Amino acid metabolism (48%) was the most altered global pathway, followed by the global changes in metabolites of nucleotide metabolism (15%), xenobiotics (14%), lipids (10%), cofactors & vitamins (5%), carbohydrates (5%), peptides (2%) and energy metabolism (2%) (Fig. 1d).

Metaboanalyst assessment of KEGG pathways shows the most impacted or altered metabolism pathways found in urine from the EAE group were 1) phenylalanine, tyrosine and tryptophan biosynthesis, 2) ubiquinone and other terpenoid-quinone biosynthesis, 3) phenylalanine metabolism, 4) arginine and proline metabolism, 5) tyrosine metabolism, and 6) taurine and hypotaurine metabolism (Fig. 1e; Supplementary Table 2). Analysis of the most impacted/significant metabolite hits in each pathway revealed an overlap of 7 metabolites across four impacted (number 1, 2, 3 and 5) pathways (Fig. 2a). All the seven metabolites represent phenylalanine catabolism, with tyrosine and 4-hydroxyphenylpyruvate being the two common metabolites across the four metabolic pathways (Fig. 2a). Tyrosine is synthesized from hydroxylating the essential amino acid phenylalanine. It acts as a precursor for various neurotransmitters and hormones that include catecholamines (norepinephrine and epinephrine), melatonin and the thyroid hormones (T3 and T4) and its lower levels are associated with decreased levels of neurotransmitters and neuronal hormones (Fig. 2b). The precursor of epinephrine and norepinephrine, dopamine, is also derived from phenylalanine to tyrosine to tyramine conversion (Daubner et al. 2011). Apart from catecholamines, thyroid hormones also require tyrosine as their precursor, hence low levels of tyrosine may also result in low levels of thyroid hormones. Similarly, melatonin levels have been reported to be low in MS patients and associated with increased fatigue and sleep disorders (Melamud et al. 2012). Although phenylalanine concentration above a threshold limit can cause brain damage (phenylketonuria), it has been used in combination with vitamin B12 and other compounds, as a therapy to relieve MS symptoms (Loder et al. 2002; Wade et al. 2002). Phenylalanine CSF concentration was decreased in MS patients with active and inactive plaques and may be linked to the presence of inflammatory plaques (Lutz et al. 2007b). Phenylalanine and tyrosine are needed for gluconeogenesis and for catabolism to provide intermediates for the tricarboxylic acid cycle (TCA) cycle. A decreased tyrosine level may reduce the biosynthesis of catecholamines (such as noradrenaline). Noradrenalin has anti-inflammatory and neuroprotective properties, and its levels are reduced in MS and EAE (Polak et al. 2011). Decreased levels of phenylalanine may affect the availability of tryptophan and tyrosine and ultimately TCA cycle dysfunction. Tryptophan is critical for two downstream biosynthetic pathways including generation of 5-hydroxytryptamine (5-HT, serotonin) and formation of kynurenine derivatives and NAD. A decreased tryptophan availability may reduce the biosynthesis of 5-HT. A decrease in 5-HT levels during early life due to the deficient availability of tryptophan is postulated to increase susceptibility to MS (Gong et al. 2008). Plasma and CSF tryptophan levels are diminished in chronic MS patients and increase the susceptibility of these patients to stress (Sandyk 1996). Moreover, metabolites of kynurenine pathway (KP) of tryptophan metabolism have been reported to have a strong association with MS subtype and may be associated with the switch from early-mid stage MS to debilitating progressive forms of MS (Lim et al. 2017). Thus, the phenylalanine-tyrosine-tryptophan and the tryptophan-related metabolic pathways appear to be a significant role player in mediating the pathology of MS via hormone imbalance and may offer putative biomarkers to follow the disease progression.

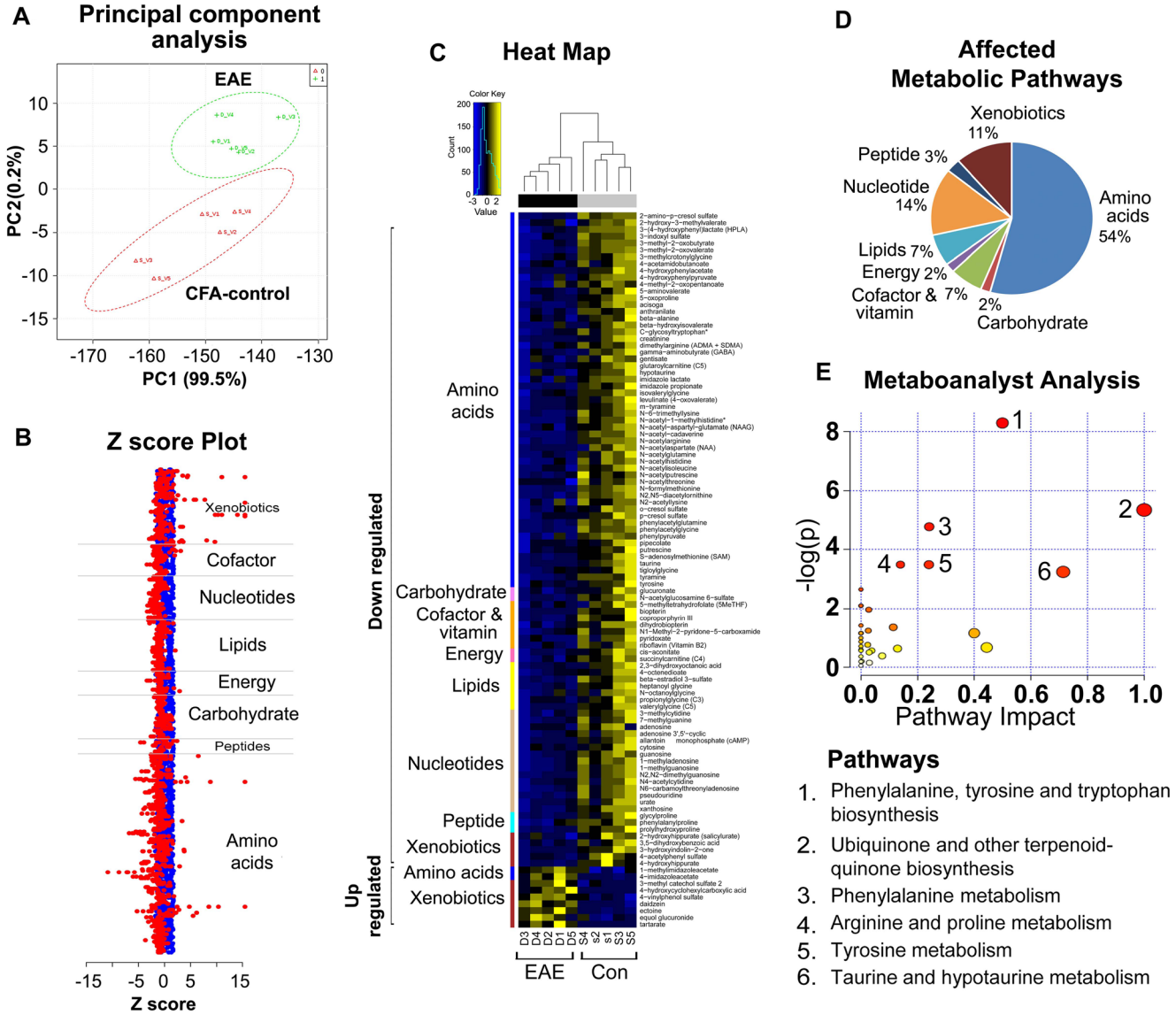

Fig. 1 Urine Metabolomic profiles of control and EAE mice at the chronic stage of the disease. **a** Principal components plot for urine samples of CFA-control versus EAE group at the chronic stage (day 45). **b** The Z-score plot of altered metabolites in urine of EAE (red) versus the CFA-only group (blue). **c** Heat map visualization for the urine of chronic stage EAE. The heat maps were constructed based on the potential candidates of importance, which were determined by two-sample t-test. **d** Pie chart summary of metabolic perturbations detected in the urine at the chronic stage of the disease. **e** Metaboanalyst analysis of KEGG metabolic library. **f** KEGG database was searched for each metabolite detected as perturbed, and each KEGG pathway scored according to the number of metabolites listed within that pathway

The levels of taurine and hypotaurine were significantly reduced in the urine of EAE compared to control group (Fig. 2c). Taurine, from the taurine-hypotaurine metabolism, is an organic metabolite derived from cysteine in the pancreas, and a major constituent of bile acid (Sumizu 1962), with hypotaurine an intermediate between cysteine and taurine. Along with its fundamental biological roles as conjugation of bile acids, antioxidation, osmoregulation and membrane stabilization, it is also considered essential for cardiovascular, skeletal muscle, retina, and CNS function (Sumizu 1962). Taurine can cross the blood-brain barrier (Urquhart et al. 1974; Tsuji and Tamai 1996) and has been implicated in participating in various physiological neurotransmission functions (Urquhart et al. 1974).

The metabolites of arginine and proline metabolism including GABA (G-aminobutyrate) and protein breakdown metabolites (putrescine and n-acetylputrecine) are decreased in the urine of the EAE group compared to control (Fig. 2d). A recent study has also reported GABA levels to be low in RRMS patients and to correlate with worse cognitive performance (Cawley et al. 2015; Cao et al. 2018). Putrescine present in a high amount, but low in EAE urine, is synthesized from arginine and belongs to a class of metabolites called polyamines. While the function of polyamines is not clear, they have been implicated in regulating protein

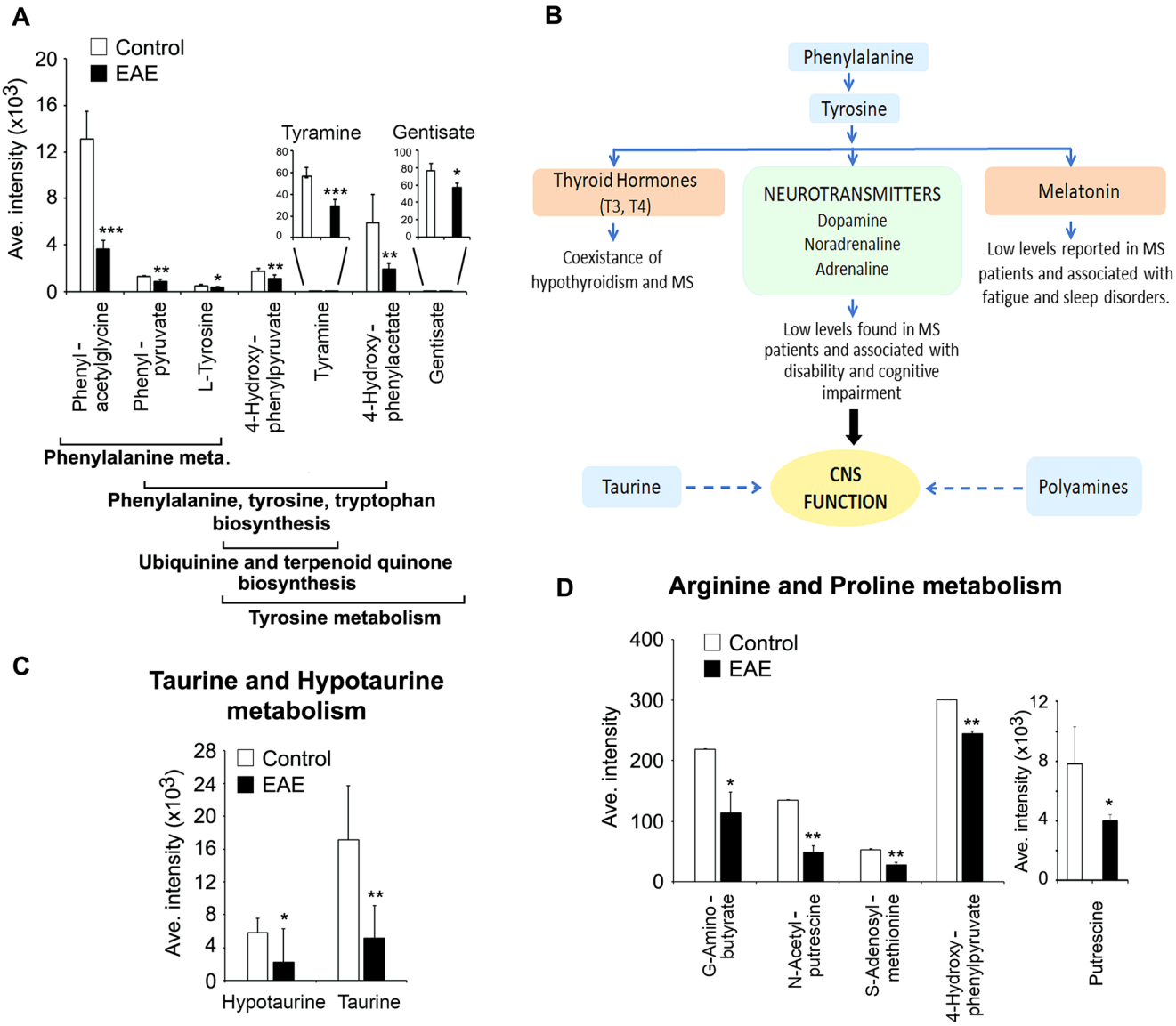

Fig. 2 Metabolite changes in the most significant and impacted metabolic pathways. **a**, **b**, **c** Average intensities representing the abundance of each metabolites belonging to various metabolic pathways are plotted as bar graphs. **d** Schematic showing the physiologic function regulated by the key altered metabolites in relation to available MS relationship. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ compared to CFA/PT control ($N = 5$)

synthesis and cell cycle. Polyamines have been shown to modulate various ion channels, including NMDA and AMPA receptors. Putrescine along with SAM (S-adenosylmethionine) results in the formation of another polyamine, spermidine. Spermidine possesses antioxidant properties and is reported to promote retinal cell survival and nerve regeneration in mice after injury (Noro et al. 2015). Spermidine alleviates EAE by inducing inhibitory macrophages through regulating the infiltration of CD4+ T cells and macrophages in the CNS (Yang et al. 2016).

A recent study by Gebregiworgis et al.,(Gebregiworgis et al. 2013) reported urinary metabolite changes at peak stage (day 17) in a chronic model of EAE mice using the 1HNMR approach and presented a six-metabolite signature representative of EAE. Our studies differed in using different analysis platforms (NMR vs. GC-MS/LC-MS in our study), and the time of sample collection (peak day 17 vs. chronic day 45 in our study). However, interestingly, some common metabolites including creatinine, taurine, phenyl acetyl glycine, 4-hydroxyphenyl pyruvic acid, 3-methyl-2-oxovalerate, and indoxyl sulfate detected by both the platforms showed a similar trend in both the studies.

Overall, our analysis shows that at the chronic stage of EAE, urine metabolomic profile of EAE and control group is significantly distinct, with the largest, significant and most impacted alterations in the metabolites of the amino acid metabolism; namely phenylalanine-tyrosine, taurine and polyamine metabolism metabolites. While the altered metabolites belonging to these impacted pathways participate in multiple physiological functions, they appear to converge at modulating CNS function by direct or indirect means (Fig. 2b). Further in-depth studies are required to assess if the metabolite

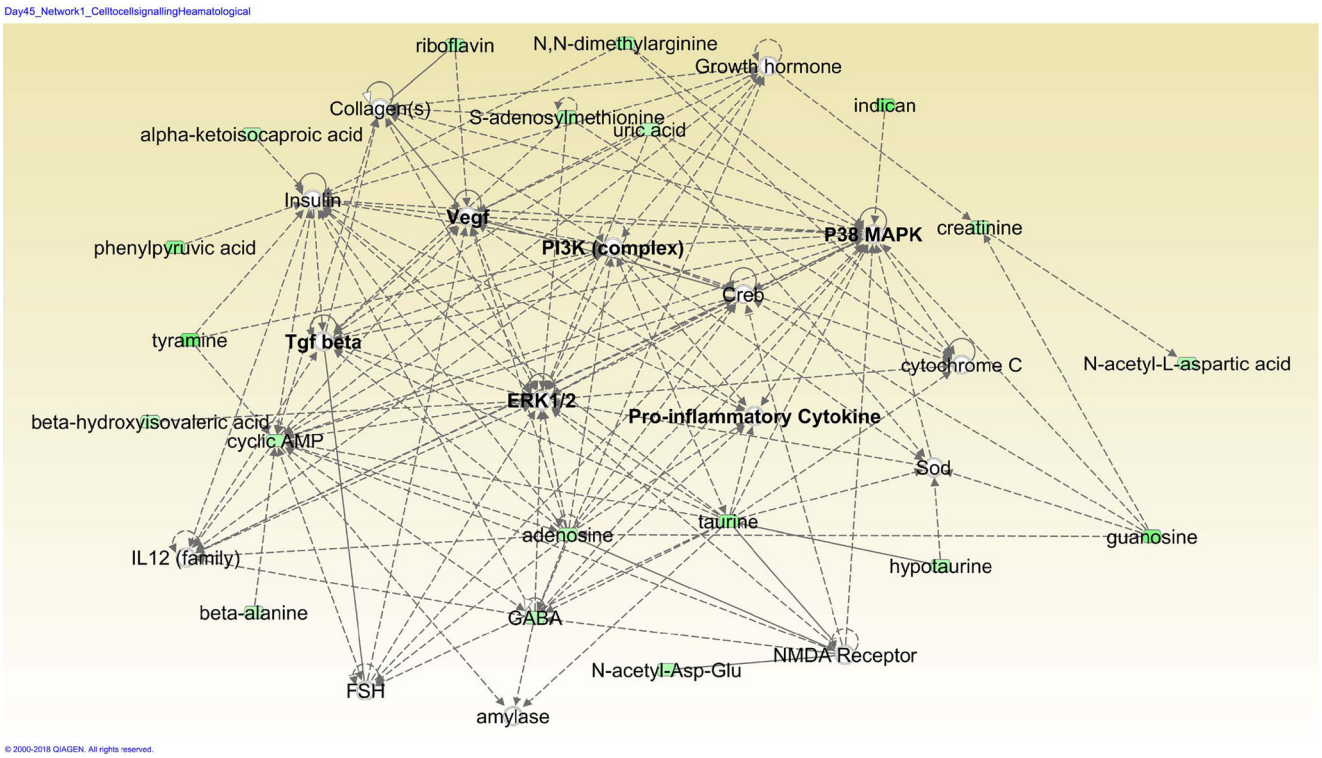

Fig. 3 Ingenuity pathway analysis (IPA). IPA of metabolites that differed significantly between urine of day 45 EAE compared to the control group after a Welch's two-sample t-test analysis. In this top network represented here over-expressed in samples from chronic stage EAE mice urine highlighted in red, and the under-expressed metabolites highlighted in green. The central most connected nodes are in bold

changes are indicative of deteriorated CNS function, or their deficiency is the cause of deteriorated CNS function. These data offer a strong rationale for validating the key metabolites (Fig. 2) along with markers of CNS function (catecholamines and thyroid hormones, for example) in patient urine to arrive at a putative biomarker for MS diagnosis as well as monitoring the disease during relapse.

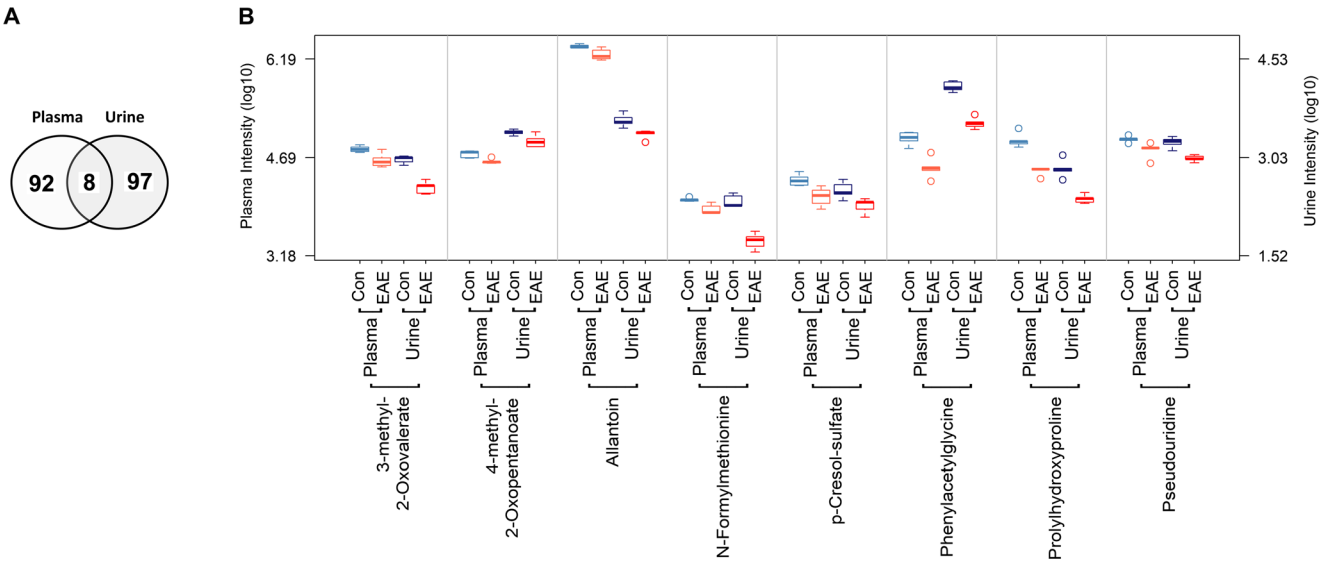

Fig. 4 Eight common metabolites were significantly altered in urine and plasma at the chronic stage of the disease. **a** Veen diagram of all significant differential metabolites in plasma and urine showed that there are eight common significantly changed metabolites. **b** Intensity values from the eight metabolites that were altered between disease and sham, in both plasma and urine, were scaled against the median intensity value. These were plotted as boxplots on a log2 scale. The axes were then back-transformed to a log10 scale for the original data and annotated on the left (plasma) and right (urine) vertical axes

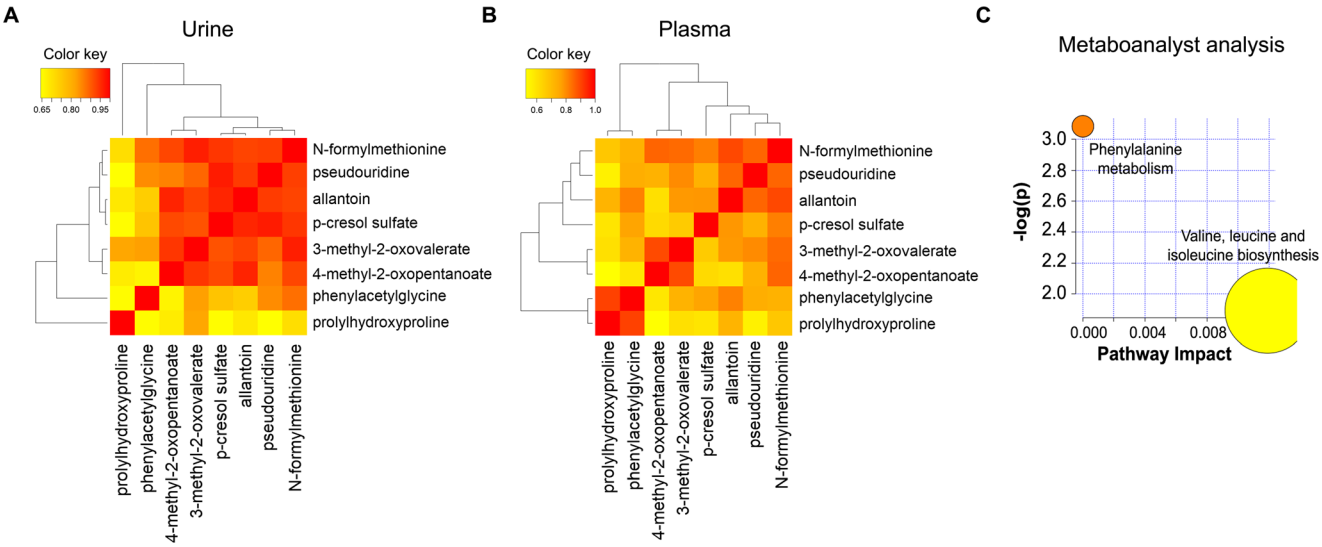

Fig. 5 Metaboanalyst analysis of significantly altered eight common metabolites in urine and plasma at the chronic stage of the disease. **a** Pearson correlation analysis of 8 metabolites in urine on day 45. **b** Pearson correlation analysis of eight metabolites in plasma at day 45. **c** Metaboanalyst analysis of significantly altered metabolites in urine and plasma at day 45

Biological Networks Associated with the Differentially Expressed Metabolites

Association of metabolites within urinary metabolite profile, beyond the boundaries of canonical pathways, may give a global insight into EAE pathology. We used Ingenuity's Pathway Analysis (IPA) to build networks from urinary metabolites that were differentially altered between EAE and control groups. The top network, involved concepts of "cell-cell signaling and interaction, hematological system development and function, and inflammatory response," and included 19 molecules differentially expressed between EAE and control urine (Fig. 3, Supplementary Table 3; Score of 45). Network 2, included nine differentially expressed molecules and focused on "molecular transport, nucleic acid metabolism, small molecule biochemistry" (Fig. S1, Suppl. Table 3; score 17). Network 3, included five differentially expressed molecules and was focused on concepts of "drug metabolism, molecular transport, small molecule biochemistry" (Fig. S2, Suppl. Table 3; Score of 13).

By expanding the metabolite Network 1 (Fig. 3) to include signaling molecules pre-defined by IPA, we show that these metabolites are interconnected by signaling molecules known to be involved in EAE/MS pathology. Namely, ERK-1/2, PI3K, p38-MAPK, VEGF. TGF beta and inflammatory cytokines along with IL12 family formed the most connected nodes. Network 2 also showed PI3K-Akt, p38 along with the prime pro-inflammatory NFkB as the central nodes. All the represented molecules have been extensively reported as abnormally altered in EAE/MS (Krementsov et al. 2013; Mc Guire et al. 2013; Birkner et al. 2017). Thus, the altered metabolites represented in the urine of the EAE group may either be responsible for mediating some of the abnormal signalings or may describe the end products of the abnormal signaling.

Commonly Altered Metabolites in Urine and Plasma of EAE

Previously, we profiled plasma metabolites at day 45 of EAE compared to the control group and identified 100 metabolites which were significantly altered in the plasma of EAE group (Poisson et al. 2015b). To detect commonly changed metabolites, present in both the biofluids we compared the metabolite profiles of urine with plasma. Eight common metabolites namely 3-methyl-2-oxovalerate, 4-methyl-2-oxopentanoate, allantoin, N-formylmethionine, p-cresol sulfate, phenylacetylglycine and pseudouridine, were observed to overlap between the urine and plasma of EAE group (Fig. 4a; Suppl. Table 4). Using Pearson correlation coefficients, we examined the correlation between these eight metabolites in plasma and in urine. A higher correlation was found in plasma resulting in tighter clusters compared to urine, but the metabolites clusters had consistent membership between the two biofluids (Fig. 5a-b, Suppl. Tables 5). These correlation plots suggest that plasma and urine metabolites are consistent. Enrichment analysis of the common eight metabolites in Metaboanalyst revealed that phenylalanine metabolism and Valine, leucine, and isoleucine biosynthetic pathways are the commonly altered metabolic pathways in both the biofluids (Fig. 5c, Suppl. Table 6).

Overall, our study defines the distinct urinary metabolic profiles between EAE and control groups. We also identify a metabolites signature of eight metabolites belonging to the phenylalanine metabolism and Valine, leucine, and isoleucine biosynthetic pathways that are altered in both plasma and

urine of chronic stage EAE mice. The eight metabolites signature may provide a non-invasive source biomarker indicating chronic progressive EAE disease risk and may be tested as markers of drug response. Recent metabolomics studies of plasma or serum from MS patients identified metabolites significantly altered with disease relative to healthy subjects (Dickens et al. 2014; Bhargava et al. 2017; Villoslada et al. 2017). Only Dickens et al. reported urinary metabolites in MS patients, thus few comparisons can be made with the pathways identified in our EAE model (Dickens et al. 2014). Among those found to be altered by Dickens et al., only creatinine was found as common with what we observed in our study (Dickens et al. 2014). We realize that EAE is not a perfect model of MS. However, EAE has proven to be instrumental in understanding the molecular events that take place upon neuroinflammation and for testing potential drugs for MS preclinical (Lovett-Racke 2017; Bjelobaba et al. 2018). Examples of successful pre-clinical therapeutic studies in EAE that have proven successful in MS patients include IFN-beta (Abreu 1982; Paty and Li 1993), GA (Teitelbaum et al. 1971; Johnson et al. 1995), and the anti-VLA-4 antibody (Yednock et al. 1992; Polman et al. 2006). The present study explores the concept that a common metabolic signature of urine and plasma may aid in the development of an objective non-invasive monitoring method for progressive autoimmune diseases like MS. This signature will need to be validated in MS patients prior to clinical use.

Acknowledgements The study was supported in part by funds from the Henry Ford Health System internal funding (A20020) and a research grant from the National Multiple Sclerosis Society (RG 4311A4/4) to SG.

Compliance with Ethical Standards

Conflict of Interest The authors have no conflict of interest.

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