Supplementary material

The potential of urinary metabolites for diagnosing multiple sclerosis

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Supplementary Discussion

For multiple sclerosis (MS), biomarkers are expected to be related to inflammation, axonal damage, demyelination, oxidative stress, and remyelination. As a result, a number of cellular and protein biomarkers have been proposed[1-4] that include: (i) cytokines and their receptors [interleukin (IL)-6, IL-10, IL-12], (ii) chemokines and their receptors (CCR5, CXCR3, CXCL10), (iii) antibodies [anti-myelin basic protein (a-MBP), anti-myelin oligodendrocyte glycoprotein (MOG)] (iv) antigen-processing and presentation [CD40,CD40L, heat shock protein], (v) cell-cycle and apoptosis (c-FLICE inhibitory protein, tumor necrosis factor-related apoptosis-inducing ligand), (vi) cellular subpopulations ($CD4^{+}/CD25^{bright}$ T cells, NK cells, NKT cells), (vii) demyelination (QYNAD peptide), (viii) axonal/neuronal damage (NF-L, Tau protein), and (ix) remyelination (neural cell adhesion molecule, ciliary neurotrophic factor). For obvious reasons, the search for MS biomarkers has focused on the analysis of cerebrospinal fluid (CSF).[5-15] Unfortunately, this endeavor has proven to be extremely challenging and none of these proposed biomarkers have been successful to date.[1-4] Additionally, there are associated risks with obtaining CSF from patients that diminishes its value as a routine diagnostic tool.[16, 17] Conversely, the analysis of urine for MS biomarkers has been minimally explored, where the focus has been on the analysis of specific metabolites, neopterin, nitric oxide and p-cresol sulfate, as surrogates for interferon-β-1 or a-MBP-like material.[18-20] A number of metabolites identified by our in-depth NMR analysis of urine from experimental autoimmune encephalomyelitis (EAE), EAE-treated, and healthy mice have been previously described in the literature of having an association with EAE, MS or neurological diseases. A brief summary of these prior findings is presented.

Urea concentrations are increased in the serum in EAE mice, which is probably a result of kidney dysfunction that is caused by EAE.[21] Renal problems have also been observed in MS patients.[22] Bireley *et al.* demonstrated a relationship between alterations in the urea cycle and neurological disorders.[23] Similarly, Toncev *et al.* observed a decrease in serum uric acid levels in MS patients.[24] EAE has also been shown to be inhibited by urea and by drugs used for treating urea cycle disorder. Specifically, sodium benzoate[25] and sodium phenyl acetate[26] were shown to ameliorate the severity of EAE.

Taurine has been reported to have neuromodulation, immunomodulation, and neuroprotective effects.[27] Taurine derivatives, such as acamprostate and taurine chloramine, also can modulate lymphocyte proliferation, cytokine production, leukocyte activation, and dendritic cell function in *in vivo* experiments.[28, 29] Taurine has been shown to increase in the CSF from EAE rats[30], tissues from EAE mice [31], and CSF from MS patients.[32] Taurine analogs have been shown to ameliorate the severity of EAE.[33]

3-hydroxyisobutyric acid is an intermediate of valine metabolism.[34] Accumulation of 3-hydroxyisobutyric acid in tissues results in a corresponding increase in urinary excretion and is shown to be associated with brain damage and neurodevelopmental problems.[35-37] An *in vitro* study also demonstrated that 3-hydroxyisobutrate inhibits enzymes involved in energy metabolism in the cerebral cortex of young rats.[38]

A mutation in the human *ETHE1* gene (ethylmalonic encephalopathy protein 1) is characterized by lesions in the basal ganglia and brainstem with increased levels of **ethylmalonic acid** in body

fluids.[39] Alternatively, short-chain acyl-CoA dehydrogenase deficiency (SCAD) is also known to increase the amount of ethylmalonic acid in the urine.[40] Among other symptoms, SCAD causes seizures and epilepsy.

3-ureidopropionate is a product of pyrimidine degradation. Pyrimidine metabolism abnormalities are reported to be associated with neurological diseases.[41]

Guanidinoacetate is an intermediate in the biosynthesis of creatine and has been shown to be associated with neurological disorders due to a deficiency in guanidinoacetate methyltransferase.[42]

Agmatine is an intermediate of arginine metabolism.[43] **Creatine** metabolism depends on arginine, where a decrease in creatine contributes to neurological symptoms.[44] Creatine was also shown to be increased in the white matter from patients with relapsing-remitting multiple sclerosis.[45]

Fructose has been shown to be increased in CSF samples collected from MS patients with or without inflammatory brain plaques,[46] and from secondary progressive MS patients.[47] Furthermore, there is a close correlation between **glucose** and fructose concentrations in CSF.[48]

Histamine and its receptors (H1 to H4) have been implicated in MS pathogenesis and EAE.[49] Histamine regulates a number of physiological processes including inflammation and immune responses.

Acetylglutamic acid is a metabolic product of **glutamic acid** (glutamate), where glutamate and its receptors have been implicated in MS/EAE pathogenesis.[50] Glutamate has been shown to be either increased or unchanged in CSF from MS patients, but CSF from EAE rats had decreased levels of glutamate.[30]

Supplementary Methods

Peptide synthesis. MOG 35-55 (MEVGWYRSPFSRVVHLYRNGK) and ovalbumin (OVA) 323-339 (ISQAVHAAHAEINEAGR) were synthesized on 9-fluorenylmethyloxy-carbonyl chemistry (Neopeptide, Cambridge, MA) to a purity of more than 90% as verified by HPLC and mass spectroscopy. The peptides were dissolved in 1x phosphate buffered saline, and stored at -20^0C until used. The MOG 35-55 peptide was used to induce EAE in mice and the OVA 323-339 was used as a control.

Histopathology

Upon termination, the mice were euthanized and brains and spinal cords were collected in 10% phosphate buffered formalin. Following fixation, brain sections through cerebrum, hippocampus, cerebellum and brain-stem; and spinal cords sections comprised of three sections each from cervical, thoracic, lumbar and sacral regions were made. The tissues were stained by hematoxylin and eosin staining, blinded to treatment and examined histologically by a board certified pathologist and scored for lesion types and severity, and counts were added together. Inflammation was primarily classified as lymphocytic, suppurative, or mixed.[51, 52]

T cell proliferation assay

Lymph nodes (LN) were harvested upon termination of experiment from all the groups of mice used in the study and lymph node cells (LNC) were prepared. The cells were stimulated with MOG 35-55 and OVA 323-339 (control) peptide at a cell density of 5 x 10^6 cells/ml for two days in growth medium containing RPMI medium supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, 4 mM L-glutamine, 1x each of nonessential amino acids and vitamin mixture and 100 U/ml penicillin–streptomycin (Lonza, Walkersville, MD). Cultures were then pulsed with tritiated ^{3}[H] thymidine (1 μCi/well; MP Biomedicals, Solon, OH); 16 h later the proliferative responses were measured as counts per minute (cpm) using a Wallac liquid scintillation counter (PerkinElmer, Waltham, MA).[52]

Major histocompatibility complex (MHC) class II dextramer staining

To determine the frequencies of antigen-specific CD4 T cells in EAE vs. EAE-treated groups, we performed MHC class II dextramer staining. Briefly, soluble MHC class II/IA^b monomers covalently tethered to MOG 35-55 and IA^s /Theiler's murine encephalomyelitis virus (TMEV) 70-86 (control) were expressed in the baculovirus system and the respective dextramers were derived as described previously.[52, 53] LNC obtained from the EAE and EAE-treated mice were stimulated with MOG 35-55 (20 μg/ml) for two days in growth medium and the cells were then maintained in growth medium containing IL-2. Viable lymphoblasts harvested on day 6 poststimulation were stained with MOG 35-55 and TMEV 70-86 dextramers followed by staining with anti-CD4 (ebioscience, San Diego, CA) and 7-aminoactinomycin-D (7-AAD; Invitrogen, Carlsbad, CA). After washing, the cells were analyzed by flow cytometry

(FACSCalibur, BD, Biosciences, San Diego, CA) and the percentages of dextramer positive ($dext^+$) cells were determined in the live ($7\text{-}AAD^-$) CD4 population.[53]

Diet Control. To evaluate the impact of the supplemental DietGel on urinary metabolites, urine was collected from healthy mice that received either the Teklad global 16% protein rodent diet (n=10) or the Teklad global 16% protein rodent diet supplemented with DietGel (n=10). Twenty 6 to 8-week-old female C57Bl/6 ($H\text{-}2^b$) mice were obtained from the Jackson Laboratory (Bar Harbor, ME). The mice were randomly classified into four cages and were acclimatized for three days before the start of the experiment. For seven days, the mice received the Teklad global 16% protein rodent diet with or without the supplemental DietGel. Urine samples were collected on the, fifth, sixth and seventh days of the experiment. Urine collections occurred three times daily (10-11 AM; 2-3 PM and 10-11 PM) from each animal by expressing the bladder. The urine samples collected from individual animals were preserved as separate aliquots and stored at -80^o C until further analysis. NMR data collection analysis and the follow-up statistical analysis followed the identical protocol described for the EAE induction and treatment experiment.

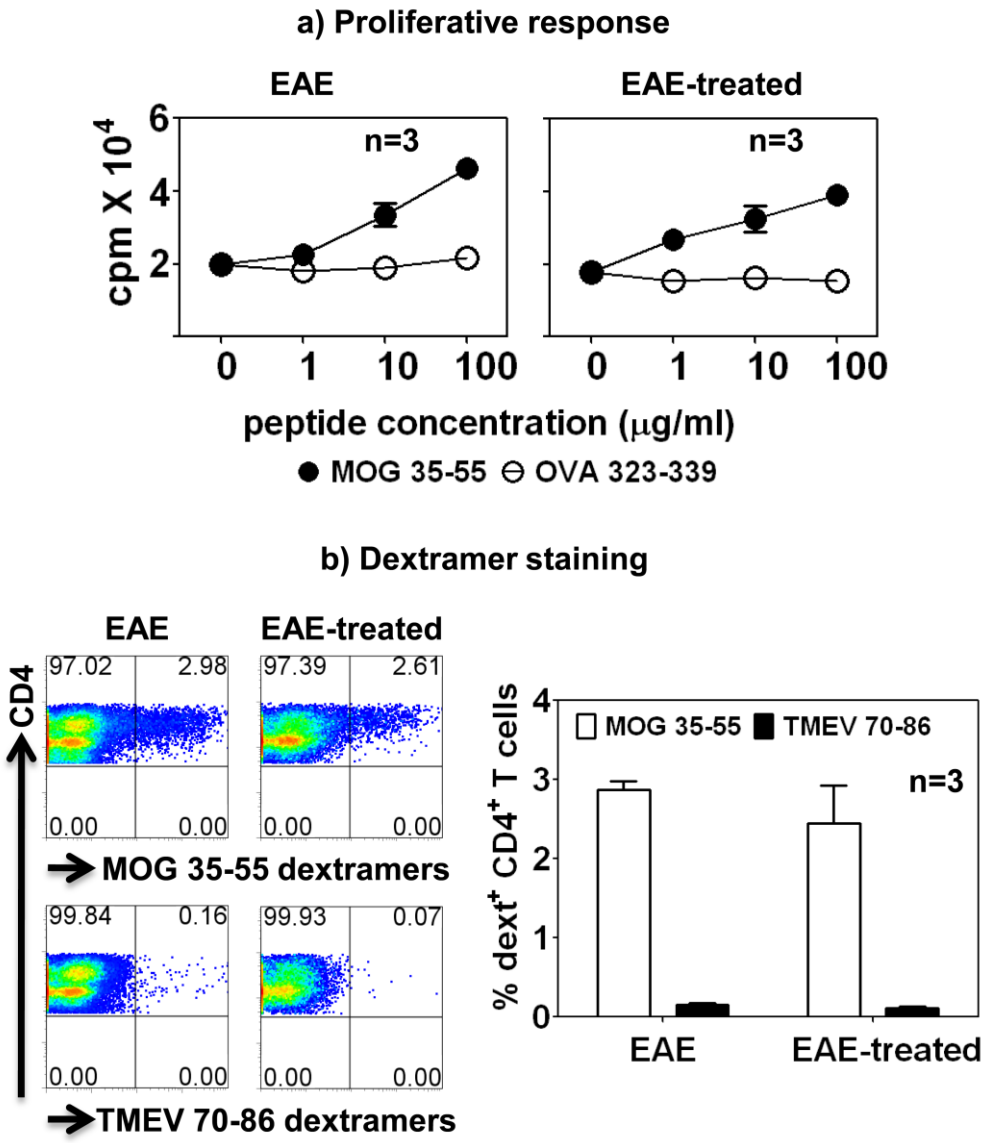
Supplementary Table and Figures

Supplementary Table 1. Histological evaluation of brains and spinal cords

Treatment	Clinical disease			No. of inflammatory foci[a]		
	Incidence (%)	Mean day of onset[b]	Mean maximum score[b]	Meninges	Parenchyma	Total
Saline				0	0	0
Saline-treated				0	0	0
CFA				0	0	0
CFA-treated				0	0	0
EAE	13/13 (100)	13.5 ± 0.4	3.5 ± 0.3	6.54 ± 1.26	7.53 ± 1.37	14.07 ± 2.47
EAE-treated	1/13 (7.69)			0.04 ± 0.04	0.04 ± 0.03	0.08 ± 0.06
p-values				$8.3x10^{-6}$	$1.8x10^{-5}$	$5.2x10^{-6}$

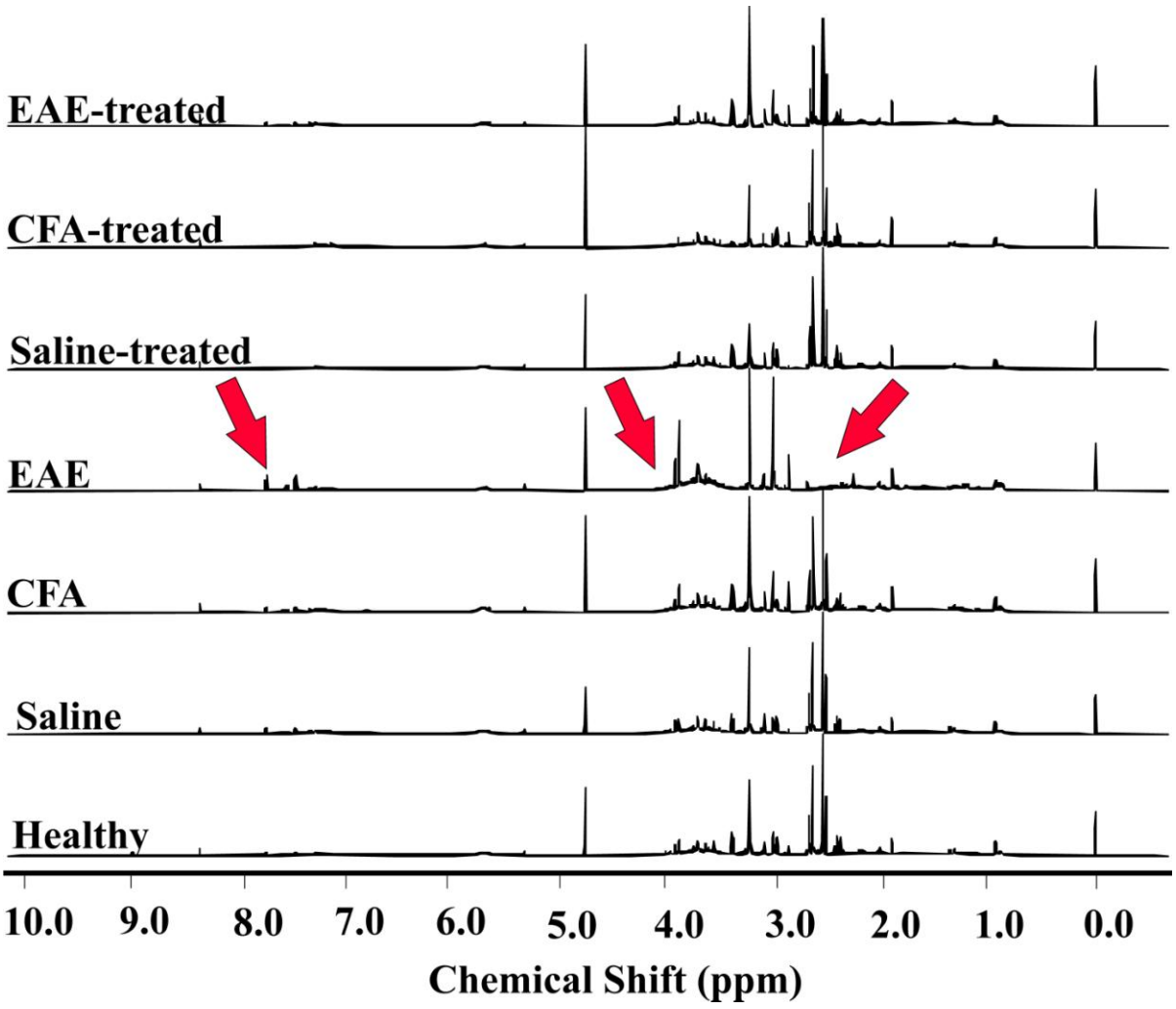
[a] numbers are mean ± SEM. [b] represents only mice that showed clinical disease.

Scoring scale: 0, no signs of disease; 1, limp tail or hind limb weakness; 2, limp tail and hind limb weakness; 3, partial paralysis of hind limbs; 4, complete paralysis of hind limbs and 5, moribund or dead**.**

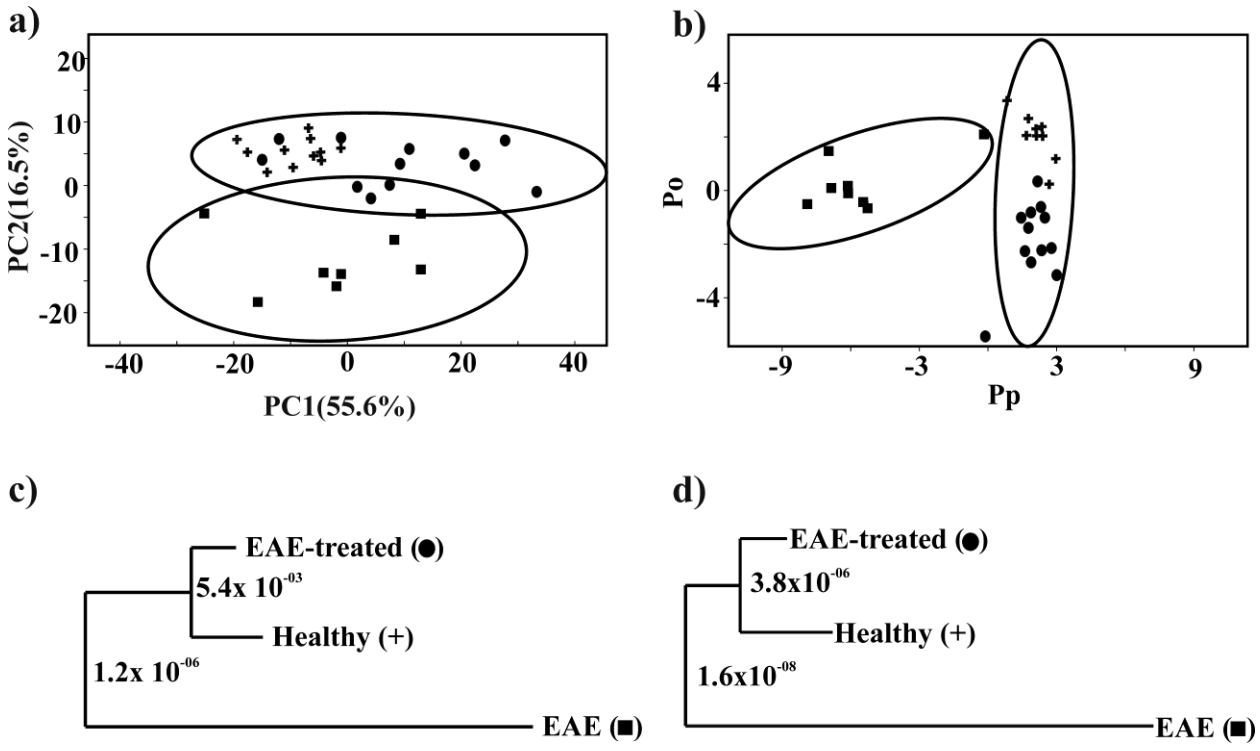


Supplementary Figure 1. (a) T-cell proliferative response. Groups of mice were immunized with MOG 35-55 in CFA, and the animals were treated with or without fingolimod daily (1mg/kg body weight) starting day 7 postimmunization until day 30. At termination, LN were harvested to prepare single cell suspensions. LNC were stimulated with MOG 35-55 and OVA 323-339 (control) for two days, and after pulsing with ^{3}[H] thymidine for 16 hours, proliferative responses were measured as cpm based on thymidine-incorporation. Mean ± SEM values for a

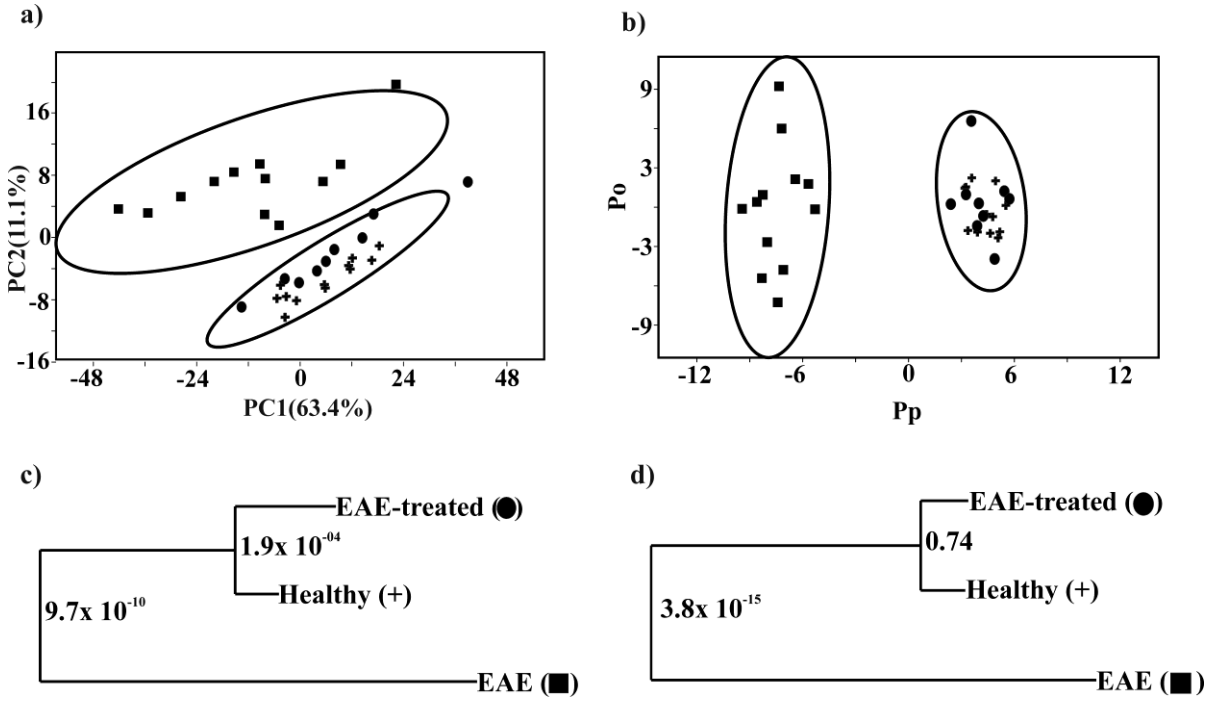
group of mice are shown. **(b) Dextramer staining.** LNC obtained from the above groups were stimulated with MOG 35-55 (20µg/ml) for two days and the cells were maintained in growth medium containing IL-2. On day 6 poststimulation, viable lymphocytes were stained with IA^b/MOG 35-55 and IA^s/TMEV 70-86 (control) dextramers, anti-CD4 and 7-AAD. After acquiring the cells by flow cytometry, percentages of $dext^+$ cells were analyzed in the live (7-AAD^-) CD4 subset. Left and right panels represent flow cytometric dot plots and mean ± SEM values respectively.



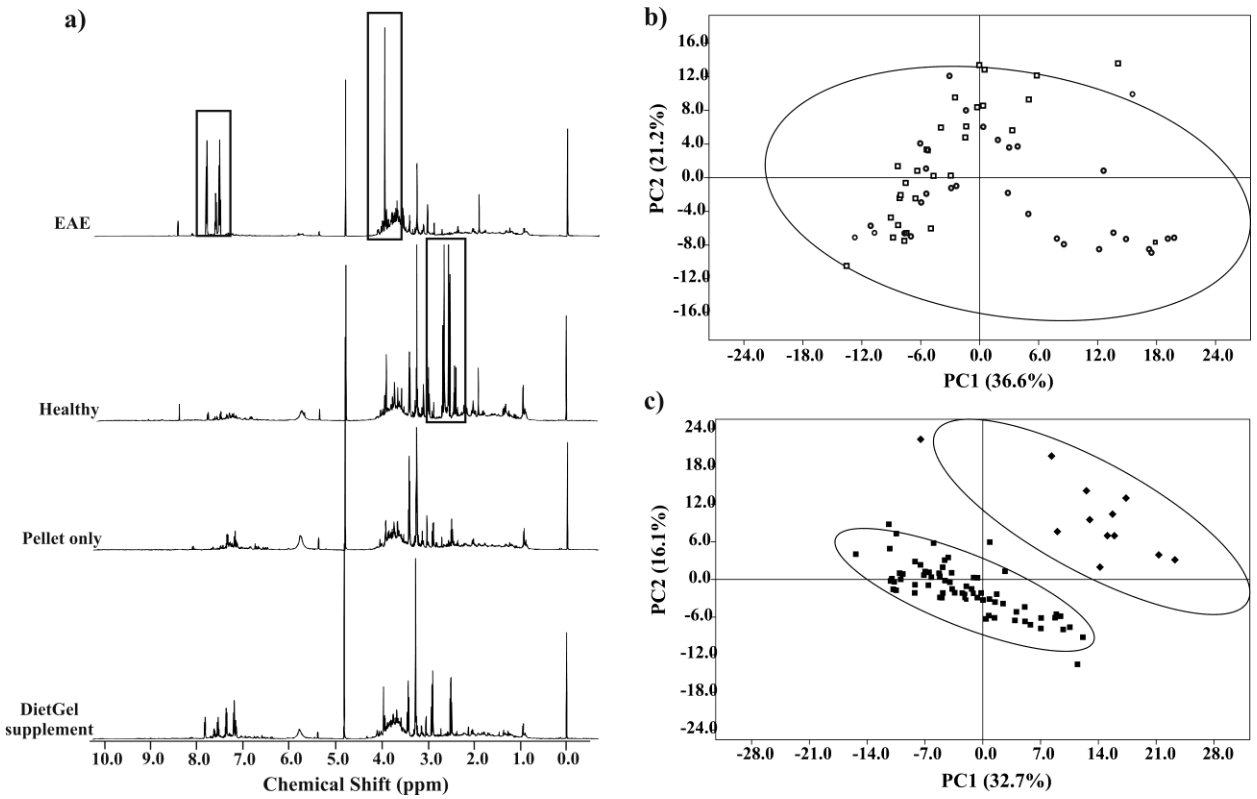
Supplementary Figure 2. Representative 1D ^{1}H NMR spectra of urine samples obtained from healthy, saline, CFA, EAE, saline-treated, CFA-treated and EAE-treated mice. The red arrows indicated regions of the spectra with visible alterations in EAE group in comparison with the other groups.



Supplementary Figure 3. (a) 2D PCA and (b) 2D OPLS-DA scores plot generated from the 1D ^{1}H NMR spectra acquired for the urine samples from day 23. The three classes are healthy (+), EAE (■), and EAE-treated mice (●). The OPLS-DA used one predictive component and one orthogonal component to yield an R^2X of 0.761, R^2Y of 0.947 and Q^2 of 0.853. The CV-ANOVA validation of the OPLS-DA class distinctions yielded a *p*-value of $3.8x10^{-7}$. The ellipses correspond to the 95% confidence limits from a normal distribution for each cluster. (c) and (d) Metabolomics tree diagrams determined from the (a) PCA and (b) OPLS-DA scores plot, respectively. The *p*-values for each node are indicated on the tree diagram.



Supplementary Figure 4. (a) 2D PCA and (b) 2D OPLS-DA scores plot generated from the 1D ^{1}H NMR spectra acquired for the urine samples from day 30. The three classes are healthy (+), EAE (■), and EAE-treated mice (●). The OPLS-DA used one predictive component and one orthogonal component to yield an R^2X of 0.76, R^2Y of 0.97 and Q^2 of 0.922. The CV-ANOVA validation of the OPLS-DA class distinctions yielded a *p*-value of $3.8x10^{-7}$. The ellipses correspond to the 95% confidence limits from a normal distribution for each cluster. (c) and (d) Metabolomics tree diagrams determined from the (a) PCA and (b) OPLS-DA scores plot, respectively. The *p*-values for each node are indicated on the tree diagram.



Supplementary Figure 5. (a) Calculated median 1D ^{1}H NMR spectra of urine samples from EAE (n=13) and healthy mice (n=12) on day 17 from the prior EAE induction and treatment experiment (Figure 1b), healthy mice receiving Teklad global 16% protein rodent diet (days 5, 6, and 7, n=28, pellet only), healthy mice receiving the DietGel supplement (days 5, 6, and 7, n=30, DietGel supplement); (b) 2D PCA scores plot generated from the 1D ^{1}H NMR spectra acquired for the urine samples collected on days 5, 6 and 7 from healthy mice fed with Teklad global 16% protein rodent diet (□), and healthy mice that received the DietGel supplement (○).The ellipse corresponds to the 95% confidence limit from a normal distribution for the DietGel supplement cluster. (c) 2D PCA scores plot for the urine samples collected from the healthy groups representing days 5, 6 and 7 or 17 that received food pellets and/or DietGel (■), and day 17 EAE

mice (♦). The 1D ^{1}H NMR data from the prior EAE induction and treatment experiment were normalized to the 1D ^{1}H NMR data from the diet control experiment to ensure the two-sets of healthy controls overlapped in the 2D PCA scores plot. The ellipses correspond to the 95% confidence limits from a normal distribution for each cluster.

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