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# Plasma and Fecal Metabolite Profiles in Autism Spectrum Disorder

Brittany D. Needham, Mark D. Adame, Gloria Serena, Destanie R. Rose, Gregory M. Preston, Mary C. Conrad, A. Stewart Campbell, David H. Donabedian, Alessio Fasano, Paul Ashwood, and Sarkis K. Mazmanian

## ABSTRACT

**BACKGROUND:** Autism spectrum disorder (ASD) is a neurodevelopmental condition with hallmark behavioral manifestations including impaired social communication and restricted repetitive behavior. In addition, many affected individuals display metabolic imbalances, immune dysregulation, gastrointestinal dysfunction, and altered gut microbiome compositions.

**METHODS:** We sought to better understand nonbehavioral features of ASD by determining molecular signatures in peripheral tissues through mass spectrometry methods (ultrahigh performance liquid chromatography–tandem mass spectrometry) with broad panels of identified metabolites. Herein, we compared the global metabolome of 231 plasma and 97 fecal samples from a large cohort of children with ASD and typically developing control children.

**RESULTS:** Differences in amino acid, lipid, and xenobiotic metabolism distinguished ASD and typically developing samples. Our results implicated oxidative stress and mitochondrial dysfunction, hormone level elevations, lipid profile changes, and altered levels of phenolic microbial metabolites. We also revealed correlations between specific metabolite profiles and clinical behavior scores. Furthermore, a summary of metabolites modestly associated with gastrointestinal dysfunction in ASD is provided, and a pilot study of metabolites that can be transferred via fecal microbial transplant into mice is identified.

**CONCLUSIONS:** These findings support a connection between metabolism, gastrointestinal physiology, and complex behavioral traits and may advance discovery and development of molecular biomarkers for ASD.

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Many diseases are associated with informative metabolic signatures, or biomarkers, that enable diagnoses, predict disease course, and guide treatment strategies. In contrast, autism spectrum disorder (ASD) is diagnosed based on observational evaluation of behavioral symptoms, including reduced social interaction and repetitive/stereotyped behaviors (1). The average age of ASD diagnosis is between 3 and 4 years old (2), at which time children can receive behavioral therapy, the gold standard treatment. Because earlier diagnosis improves efficacy of behavioral therapies (3,4), molecular biomarkers represent an attractive approach for identifying at-risk populations and may aid development of personalized therapies. This prospect is increasingly important given the rising rate of ASD diagnoses, which currently stands at up to 1 in 59 children in the United States (2), with high variability of worldwide estimates (approximately 1%–2%) (5,6) and no Food and Drug Administration–approved drugs for core behavioral symptoms.

Metabolic abnormalities have been reported in ASD (7), though most studies have measured a small subset of metabolites, and many outcomes have not been reproduced between cohorts. Mitochondrial dysfunction, which heavily influences systemic metabolism, is estimated to be higher in ASD individuals than control subjects (5% vs. approximately 0.01%) (8), and genes crucial for mitochondrial function are risk factors for ASD (9). The metabolic abnormalities associated with mitochondrial dysfunction in ASD affect cellular energy, oxidative stress, and neurotransmission in the gut and the brain (9–21). Other metabolic profiles in ASD implicate aromatic and phenolic metabolites, including derivatives of nicotinic acid, amino acid, and hippurate metabolism (22–32). Various amino acids are detected at differential levels across studies and across sample types, but consistent patterns are difficult to discern (17,26,28,31,33–36).

Some discrepancies between studies are likely due to differences in sample number, tissue analyzed, and methodology. Other sources of variability include differences in environmental factors, such as diet and gut bacteria, which differ between ASD and typically developing (TD) populations and can influence each other (37–39). Diet is a major source of circulating metabolites, impacting the metabolome directly or indirectly through chemical transformation by the trillions of gut microbes, the microbiome, which has been proposed to modulate complex behaviors (40–42). Such proposed environmental modulators of ASD may integrate with genetic risks





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to impact behavioral end points through the actions of small molecules produced in tissues outside the brain.

Herein, we present a comprehensive comparison of an extensive panel of identified metabolites in human plasma and feces from a large cohort of matched children with ASD and TD children. We identified differential levels of metabolites ranging from hormones, amino acids, xenobiotics, and lipids, many of which correlate with clinical behavior scores. To our knowledge, this is the first study to concurrently evaluate paired intestinal and systemic metabolomes in a high-powered analysis with a large number of identified metabolites, allowing direct associations between metabolites previously highlighted in ASD samples and discovering new metabolites of interest. These findings support the emerging concept of evaluating nonbehavioral features in the diagnosis of ASD.

## METHODS AND MATERIALS

Samples for this study, from children 3 to 12 years of age, were collected through the MIND Institute, University of California, Davis (43,44). ASD diagnosis was confirmed by trained staff using the Autism Diagnostic Observation Schedule (ADOS) and the Autism Diagnostic Interview-Revised. Diagnosis made in subjects before 2013 was based on DSM-IV. TD participants were screened using the Social Communication Questionnaire and scored within the typical range (<15). An additional evaluation to determine gastrointestinal (GI) symptoms was completed by 97 participants who also provided stool samples. GI status was determined using the GI symptom scale, based on Rome III Diagnostic Questionnaire on Pediatric Functional Gastrointestinal Disorders (45) and described in detail elsewhere (44). See Supplemental Methods in Supplement 1 for further information.

## RESULTS

### Plasma and Fecal Metabolomes Differ Between ASD and TD

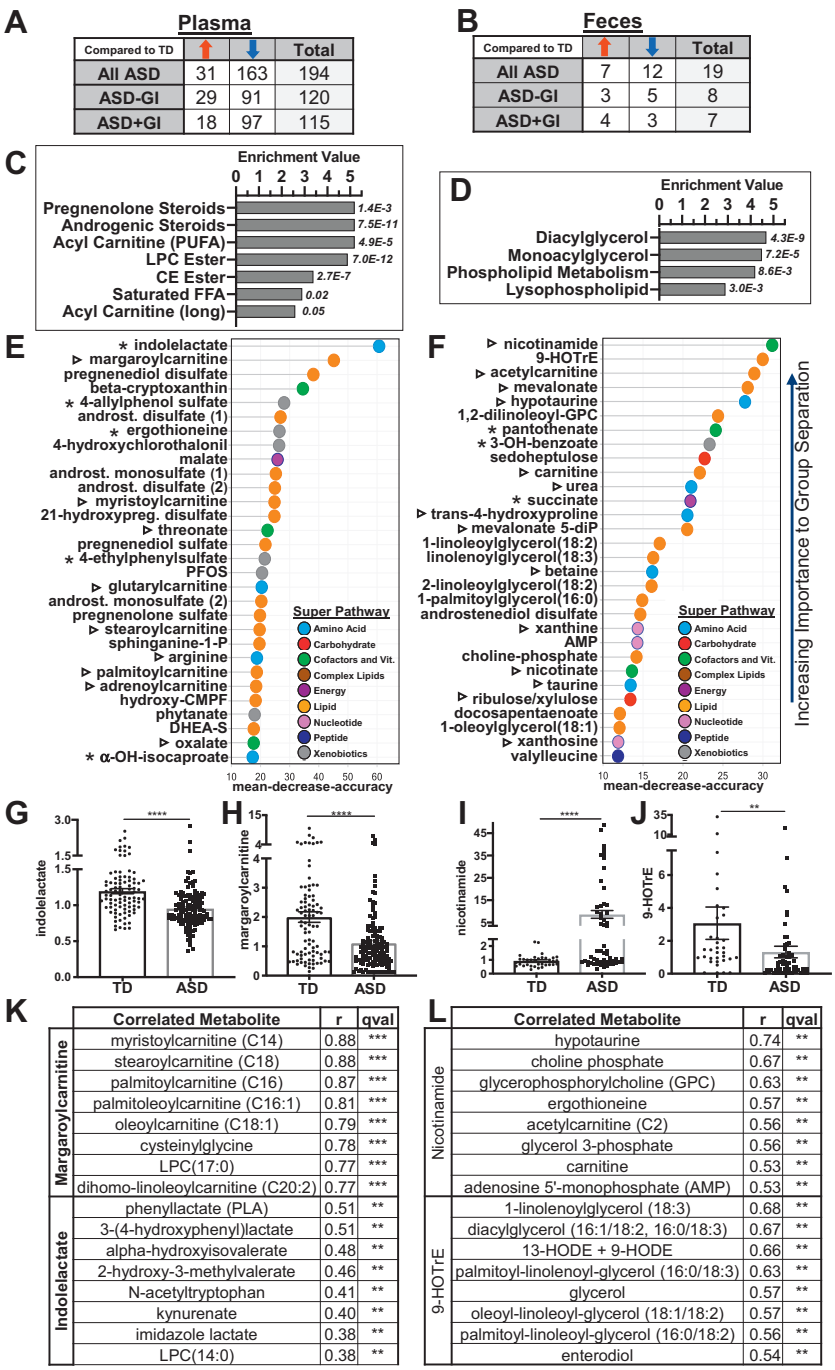
Plasma samples from 130 children with ASD and 101 TD children were analyzed along with fecal samples collected from a subset of these same children with ASD ($n$ = 57) and TD children ($n$ = 40) (Figure S1A–G in Supplement 1). Samples and metrics of behavioral and GI scores were obtained from the MIND institute (43,44). The ASD group was stratified into subsets of children with GI symptoms (ASD+GI) or without GI symptoms (ASD−GI), to explore potential effects of comorbid intestinal dysfunction in ASD (40 of 130 ASD samples were ASD+GI). This stratification was based on symptoms associated with ASD, including diarrhea, constipation, and irritable bowel syndrome–like symptoms.

Samples were analyzed by the global metabolite panel (plasma and fecal samples) and the complex lipid panel (plasma samples) from Metabolon, Inc. (Morrisville, NC), which identified a total panel of 1611 plasma and 814 fecal metabolites (Tables S1–S6 in Supplement 2). Overall, we discovered that 194 metabolites were differentially abundant between the ASD and TD groups in plasma and 19 metabolites were differentially abundant in feces (Figure 1A, B; Tables S1 and S4 in Supplement 2). Using a quantitative assay for a targeted panel of metabolites, we observed a high correlation between relative abundance and precise concentration ($r^2$ = approximately .97) for all tested metabolites except $N$-acetylserine ($r^2$ = .63) (Figure S1H in Supplement 1). Overall, these data expand on previous evidence that the metabolomic profiles of ASD and TD populations display differences not only in the gut compartment but also in circulation, which may affect the levels of metabolites throughout the body, including the brain (46,47).

We then used random forest machine learning analysis to determine if metabolite profiles can predict without bias whether the sample came from an ASD or a TD donor. Overall, the modest predictive accuracy of this machine learning approach was 69% for plasma and 67% for feces. To test whether focusing on the most discriminating metabolites would improve the prediction, we repeated the random forest analysis using the top 30 metabolites and found that the predictive accuracy for plasma improved slightly to 70% when using all ASD samples and to 74% when using only ASD − GI samples. For fecal samples, the predictive accuracy improved to 75% using all ASD samples and to 67% using only ASD − GI samples. The top 30 metabolites, calculated by measuring the mean decrease in accuracy of the machine learning algorithm, are useful to describe the strongest drivers of overall metabolic differences between ASD and TD populations. These most discriminatory metabolites were primarily from the lipid, amino acid, xenobiotic, and cofactor/vitamin super pathways (Figure 1E, F). Several of these metabolites have been previously linked to ASD, such as steroids, bile acids, acylcarnitines, and nicotinamide metabolites (48–51). Further, multiple molecules known to be produced or manipulated by the gut microbiota also featured prominently, including 4-ethylphenyl sulfate (4-EPS), which is elevated in an ASD mouse model, and indolelactate, a microbe-derived tryptophan metabolite (Figure 1E) (52,53). The two most discriminatory molecules in plasma (Figure 1G, H) and feces (Figure 1I, J) are depicted, which are detected in almost every sample except for 9-HOTre, which has a slightly lower percent fill in ASD compared with TD samples (84% vs. 91%). Metabolites correlating most strongly with these discriminatory metabolites are closely related on a structural and metabolic level (Figure 1K, L).

### Metabolite Levels Correlate With Clinical Behavioral Scores

Using clinical metadata for children with ASD, we correlated the levels of individual metabolites to the verbal, social, and nonverbal scores of the following standard diagnostic measures, all conducted by trained health professionals: the Autism Diagnostic Interview-Revised (a parent questionnaire) and the cumulative ADOS severity score (SS) (Tables S1, S4 in Supplement 2) (1). To contextualize the biological relevance of different metabolomes between ASD and TD samples, individual metabolites were integrated into biochemical pathways for pathway enrichment analysis, revealing the degree of change within each. We identified changes mostly in lipid, xenobiotic, and nucleotide pathways associated with diverse physiological processes (Figure S2C in Supplement 1). We found that verbal and social scores primarily correlate with lipid metabolism pathways and that nonverbal scores have the




K

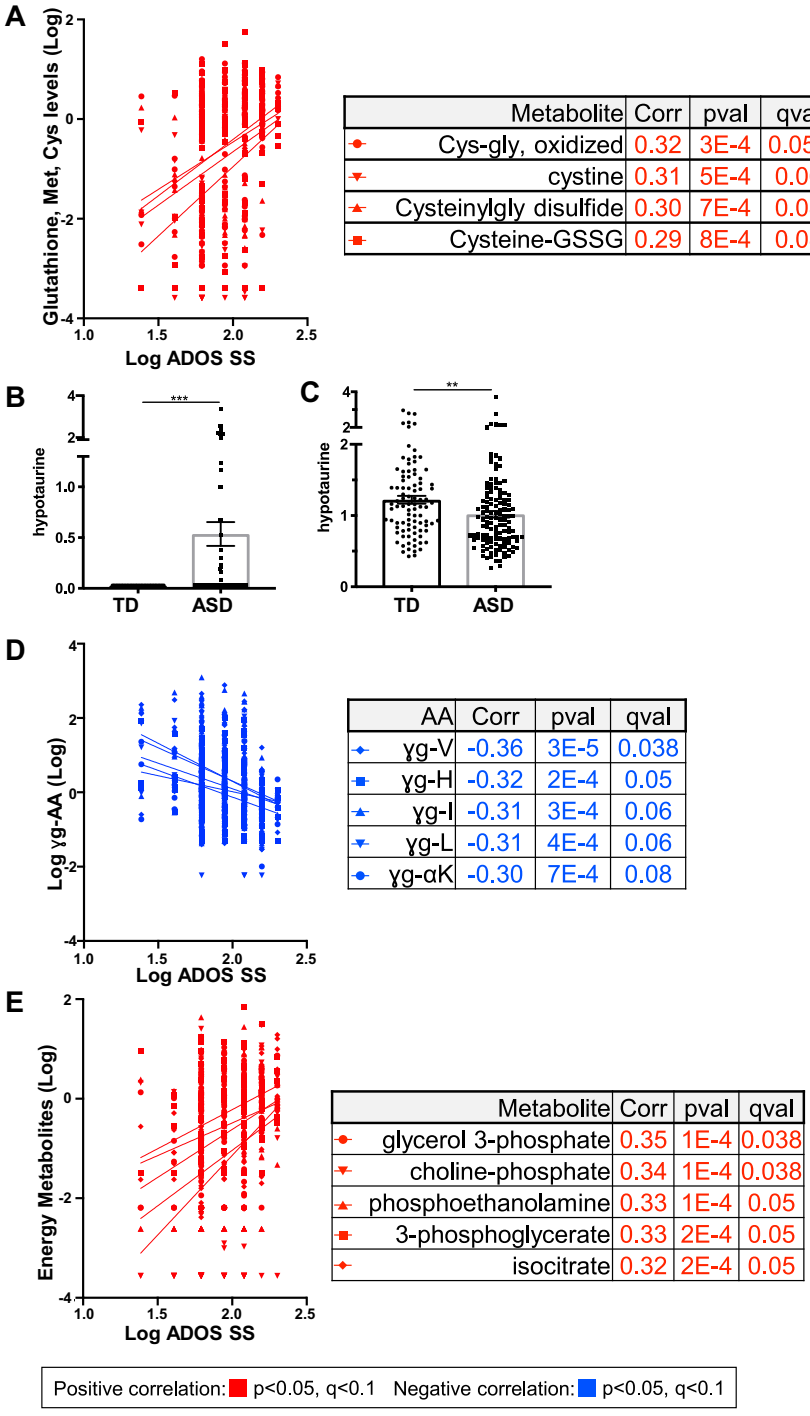
| | Correlated Metabolite | r | qval |
|---|---|---|---|
| Margaroylcarnitine | myristoylcarnitine (C14) | 0.88 | *** |
| | stearoylcarnitine (C18) | 0.88 | *** |
| | palmitoylcarnitine (C16) | 0.87 | *** |
| | palmitoleoylcarnitine (C16:1) | 0.81 | *** |
| | oleoylcarnitine (C18:1) | 0.79 | *** |
| | cysteinylglycine | 0.78 | *** |
| | LPC(17:0) | 0.77 | *** |
| | dihomo-linoleoylcarnitine (C20:2) | 0.77 | *** |
| Indolelactate | phenyllactate (PLA) | 0.51 | ** |
| | 3-(4-hydroxyphenyl)lactate | 0.51 | ** |
| | alpha-hydroxyisovalerate | 0.48 | ** |
| | 2-hydroxy-3-methylvalerate | 0.46 | ** |
| | N-acetyltryptophan | 0.41 | ** |
| | kynurenate | 0.40 | ** |
| | imidazole lactate | 0.38 | ** |
| | LPC(14:0) | 0.38 | ** |

L

| | Correlated Metabolite | r | qval |
|---|---|---|---|
| Nicotinamide | hypotaurine | 0.74 | ** |
| | choline phosphate | 0.67 | ** |
| | glycerophosphorylcholine (GPC) | 0.63 | ** |
| | ergothioneine | 0.57 | ** |
| | acetylcarnitine (C2) | 0.56 | ** |
| | glycerol 3-phosphate | 0.56 | ** |
| | carnitine | 0.53 | ** |
| | adenosine 5'-monophosphate (AMP) | 0.53 | ** |
| 9-HOTrE | 1-linolenoylglycerol (18:3) | 0.68 | ** |
| | diacylglycerol (16:1/18:2, 16:0/18:3) | 0.67 | ** |
| | 13-HODE + 9-HODE | 0.66 | ** |
| | palmitoyl-linolenoyl-glycerol (16:0/18:3) | 0.63 | ** |
| | glycerol | 0.57 | ** |
| | oleoyl-linoleoyl-glycerol (18:1/18:2) | 0.57 | ** |
| | palmitoyl-linoleoyl-glycerol (16:0/18:2) | 0.56 | ** |
| | enterodiol | 0.54 | ** |

**Figure 1.** Plasma and fecal metabolomes differ between autism spectrum disorder (ASD) and typically developing (TD) groups. **(A, B)** The number of significantly elevated and decreased metabolites ($p < .05$, $q < .1$) in ASD samples compared with the TD control group by analysis of variance contrasts in plasma and feces, respectively. Samples are stratified by all samples or samples without or with gastrointestinal (GI) symptoms (−GI, +GI). **(C, D)** Pathway analysis results of human plasma and fecal comparisons (all samples), indicating which metabolomic pathways are the most altered between groups, with enrichment value plotted and *p* value to the right of each bar. Metabolites within each pathway could be observed at either higher or lower levels, as this plot indicates only changes. **(E, F)** Top 30 most distinguishing metabolites between each group in plasma and feces by random forest analysis, with mean decrease accuracy along the x-axis, which is determined by randomly permuting a variable, running the observed values through the trees, and then reassessing the prediction accuracy. If a variable is important to the classification, the prediction accuracy will drop after such a permutation. Metabolites known to be produced by (asterisks) or influenced by (triangles) the gut microbiota are denoted. The super pathway to which each metabolite belongs is indicated by color of sphere and defined in the legend. **(G, H)** Scaled intensity values indicating relative levels of the most distinguishing molecules between ASD and TD (all samples) in plasma. Asterisks indicate significance ($p < .05$, $q < .1$) in analysis of variance contrasts performed on total metabolomics dataset. Data are represented as mean ± SEM. **(I, J)** Scaled intensity values indicating relative levels of the most distinguishing molecules between ASD and TD (all samples) in feces. Asterisks indicate significance ($p < .05$, $q < .1$) in analysis of variance contrasts performed on total metabolomics dataset. Data are represented as mean ± SEM. **(K)** Top correlated plasma metabolites that covary with margaroylcarnitine and indolelactate. **(L)** Top correlated fecal metabolites that covary with nicotinamide and 9-HOTrE. AMP, adenosine monophosphate; androst., androstane; CE, cholesterol ester; DHEA-s, dehydroepiandrosterone sulfate; FFA, free fatty acid; hydroxypreg., hydroxypregnenalone; LPC, lysophosphatidylcholine; PFOS, perfluorooctanesulfonic acid; PUFA, polyunsaturated fatty acid; qval, *q* value.

fewest correlations. Verbal scores show modest, negative correlations with levels of individual metabolites, including various plasma glycerolipids (i.e., diacylglycerols, glycerophosphorylcholine, monoacylglycerols), and both plasma and fecal levels of bile acids and sphingolipids. However, these correlations are not particularly striking on an individual level (Figure S2D in Supplement 1; Tables S1, S4 in Supplement 2). Social scores positively correlate with total free fatty acid levels in plasma, most significantly with a C18 chain length (Figure S2E in Supplement 1; Table S1 in Supplement 2). After false discovery rate correction, no individual metabolites significantly correlated with nonverbal score.




| Metabolite | Corr | pval | qval |
|---|---|---|---|
| Cys-gly, oxidized | 0.32 | 3E-4 | 0.056 |
| cystine | 0.31 | 5E-4 | 0.06 |
| Cysteinylgly disulfide | 0.30 | 7E-4 | 0.08 |
| Cysteine-GSSG | 0.29 | 8E-4 | 0.08 |

| AA | Corr | pval | qval |
|---|---|---|---|
| ɣg-V | -0.36 | 3E-5 | 0.038 |
| ɣg-H | -0.32 | 2E-4 | 0.05 |
| ɣg-I | -0.31 | 3E-4 | 0.06 |
| ɣg-L | -0.31 | 4E-4 | 0.06 |
| ɣg-αK | -0.30 | 7E-4 | 0.08 |

| Metabolite | Corr | pval | qval |
|---|---|---|---|
| glycerol 3-phosphate | 0.35 | 1E-4 | 0.038 |
| choline-phosphate | 0.34 | 1E-4 | 0.038 |
| phosphoethanolamine | 0.33 | 1E-4 | 0.05 |
| 3-phosphoglycerate | 0.33 | 2E-4 | 0.05 |
| isocitrate | 0.32 | 2E-4 | 0.05 |

**Figure 2.** Metabolite levels correlate with Autism Diagnostic Observation Schedule (ADOS) severity score (SS). **(A)** Correlation of ADOS SS with metabolites from the cysteine, methionine, and glutathione pathways. Significant metabolites corresponding to the linear regression in the graph are listed along with Spearman coefficients and *p* values. Refer to color legend at bottom. **(B, C)** Scaled intensity values indicating relative levels of hypotaurine in feces **(B)** and plasma **(C)** (all samples). Data are represented as mean ± SEM. Asterisks indicate significance in analysis of variance contrasts performed on total metabolomics dataset with a false discovery rate cutoff of $q < .1$ (**$p < .01$, ***$p < .001$). **(D)** Correlations of gamma-glutamyl amino acids with ADOS SS, with Spearman coefficients and *p* values to the right. Refer to color legend at bottom. **(E)** The top 5 most positively correlated plasma metabolites with ADOS SS, with Spearman coefficients and *p* values to the right. Refer to color legend at bottom. AA, amino acid; Corr, correlation; Cys, cysteine; Cys-gly, cysteinylglycine; GSSG, glutathione disulfide; Met, methionine; pval, *p* value; qval, *q* value.

The ADOS SS correlated with diverse metabolite pathways, including amino acids and food/plant component pathways (Figure S2C in Supplement 1), which may be partly due to the diverse array of symptoms integrated into the ADOS SS score. On an individual metabolite level, we observed significant positive correlations and trends between the ADOS SS and metabolites in pathways of oxidative stress (cysteine, methionine, *S*-adenosyl-L-methionine, and glutathione pathways) (Figure 2A; Table S1 in Supplement 2). Some of these molecules were found in higher levels in the ASD fecal samples and at lower levels in the ASD plasma samples, such as hypotaurine and, to a lesser degree, taurine (Tables S1 and S4 in

Supplement 2; Figure 2B, C; Figure S3A, B in Supplement 1), which might indicate altered fecal production, excretion, or differential uptake into the plasma potentially through varied intestinal permeability. Taurine plays many roles throughout the host and has previously been measured at altered levels in ASD, although with little consensus (19,20,26–28,35,54–56). Hypotaurine and taurine deficiency has been shown to lead to defects in cell differentiation in the brain (56), and their dysregulation could alter neuronal signaling (57).

Dysregulated amino acid degradation, homeostasis, and import into the brain have been implicated as a cause of neuronal stress in ASD, and supporting metabolomic data have shown perturbations of various amino acid pathways, such as glutamate, methionine, glutathione, and gamma-glutamyl metabolites (12,19,26,28,31,35,51,58,59), some of which show associations with the ADOS SS (Table S1 in Supplement 2). Oxidative stress–related glutathione pathway precursors, gamma-glutamyl amino acids, can influence levels of neurotransmitters such as GABA (gamma-aminobutyric acid) (57,60), which is widely thought to play a role in ASD. In a recent ASD study, an experimental treatment that led to increased gamma-glutamyl amino acids and other redox pathway metabolites correlated with improved behavior metrics in children (54). Here, we observed significant negative correlations and trends between many gamma-glutamyl amino acids and the ADOS SS (Figure 2D; Table S1 in Supplement 2). We also observed some perturbations in the urea cycle, which processes the amino group of amino acids for excretion in urine (Figure S3C, D in Supplement 1). Abnormalities in this pathway can be indicative of altered amino acid degradation observed in ASD and can lead to neurotoxic accumulation of nitrogen-containing compounds in the blood (61).

Additionally, the 5 plasma metabolites most positively correlated with ADOS SS all are involved in cellular energy pathways (Figure 2E; Table S1 in Supplement 2). Differences in energy markers could indicate a neurodevelopmental phenotype during periods when the high lipid and energy requirement in the brain is crucial (62–64). Overall, these correlations support the involvement of lipid, amino acid, and xenobiotic metabolism in the etiology of ASD, as previously described (10,50,58), and reveal new candidates for ASD biomarkers that correlate with symptom severity.

## Altered Levels of Cellular Energy and Oxidative Stress Metabolites in ASD

In line with the observed correlations between oxidative stress and cellular energy with the ADOS-SS, we further observed altered levels of related metabolites between ASD and TD samples. Metabolites that are markers of mitochondrial and oxidative stress can offer a snapshot into cellular metabolic states. These markers include acylcarnitines, which have been highlighted in various ASD studies and are established indicators of mitochondrial dysfunction (13,14,16–20,26,27,35,48,55,65–69). Acylcarnitines are formed to allow transport of lipids across the mitochondrial membranes for beta-oxidation, and abnormal levels of these conjugated lipids accumulate to higher levels with a decrease in beta-oxidation. Interestingly, high levels of short acylcarnitines are found in rodent models where ASD-like behaviors are induced with the short-chain fatty acids valproic and propionic acids (70).

We found differential levels of acylcarnitines in ASD, creating a pattern of more abundant short-chain acylcarnitines and less abundant long-chain acylcarnitines in the ASD−GI samples compared with TD samples (Figure 3A). Acylcarnitines trend toward positive correlations with more severe social behavior, an effect driven by structures with shorter moieties (C2–C14) (Figure S2C in Supplement 1; Table S1 in Supplement 2). In fecal samples, acetylcarnitine (C2) and free carnitine were elevated in ASD (Figure 3B, C) and were highly discriminatory (Figure 1F). Other mitochondrial markers in both plasma and feces were also differentially abundant in ASD (Figure 3D) along with markers of phospholipid metabolism, which occurs largely in the mitochondria and was an enriched pathway in fecal comparisons (Figures 3D and 1D). Such defects in cellular metabolism support the theory that mitochondrial dysfunction may not only be comorbid with ASD but also may be a potential contributing factor, as suggested by numerous previous reports (8,15,66,68,70), and alterations to levels of tricarboxylic acid cycle intermediates have been observed in human ASD prefrontal cortex samples (51). Together with the observed correlations between metabolites and the ADOS SS, these results corroborate and extend a growing body of research into altered mitochondrial metabolism and oxidative stress in ASD.

## Transfer of ASD Fecal Microbiota Into Mice Is Accompanied by Metabolic Signatures

As microbial metabolites ranked highly in the random forest machine learning analysis, we tested if any of the observed metabolite differences in humans could be transferred to mice via fecal microbial transplant. We selected 4 male donor samples from each of the ASD and TD groups and colonized 2 or 3 male germ-free mice per donor for 3 weeks before collecting plasma and fecal samples for metabolite profiling and bacterial DNA sequencing, respectively (Figure S4A in Supplement 1). Global metabolomic analysis revealed that colonized mice modestly cluster by donor and group when differential metabolites are considered in principal component analysis (Figure S4B in Supplement 1). We selected the human donors based on 4-EPS levels (Figure S4C in Supplement 1), owing to the involvement of 4-EPS in an ASD mouse model (52) and dysregulation of similar phenolic compounds in human ASD (24,26,30,31,71). 4-EPS is not produced by the host and is strictly a bacterial metabolite (52,72). Surprisingly, we observed 4-EPS levels in a bimodal distribution in mouse samples (Figure S4D, E in Supplement 1). Despite the surprising results with 4-EPS, many of the metabolites with the highest fold change and lowest $p$ value are indeed other phenolic molecules, such as metabolites of hippurate, tyrosine, and diet-derived phenols, some of which have been similarly measured in a previous study transferring fecal microbes from individuals with ASD into mice (Figure S4F in Supplement 1; Table S10 in Supplement 2) (73). While preliminary, these studies reveal dysregulated microbiome-mediated effects on xenobiotic pathways and phenolic metabolites in ASD.

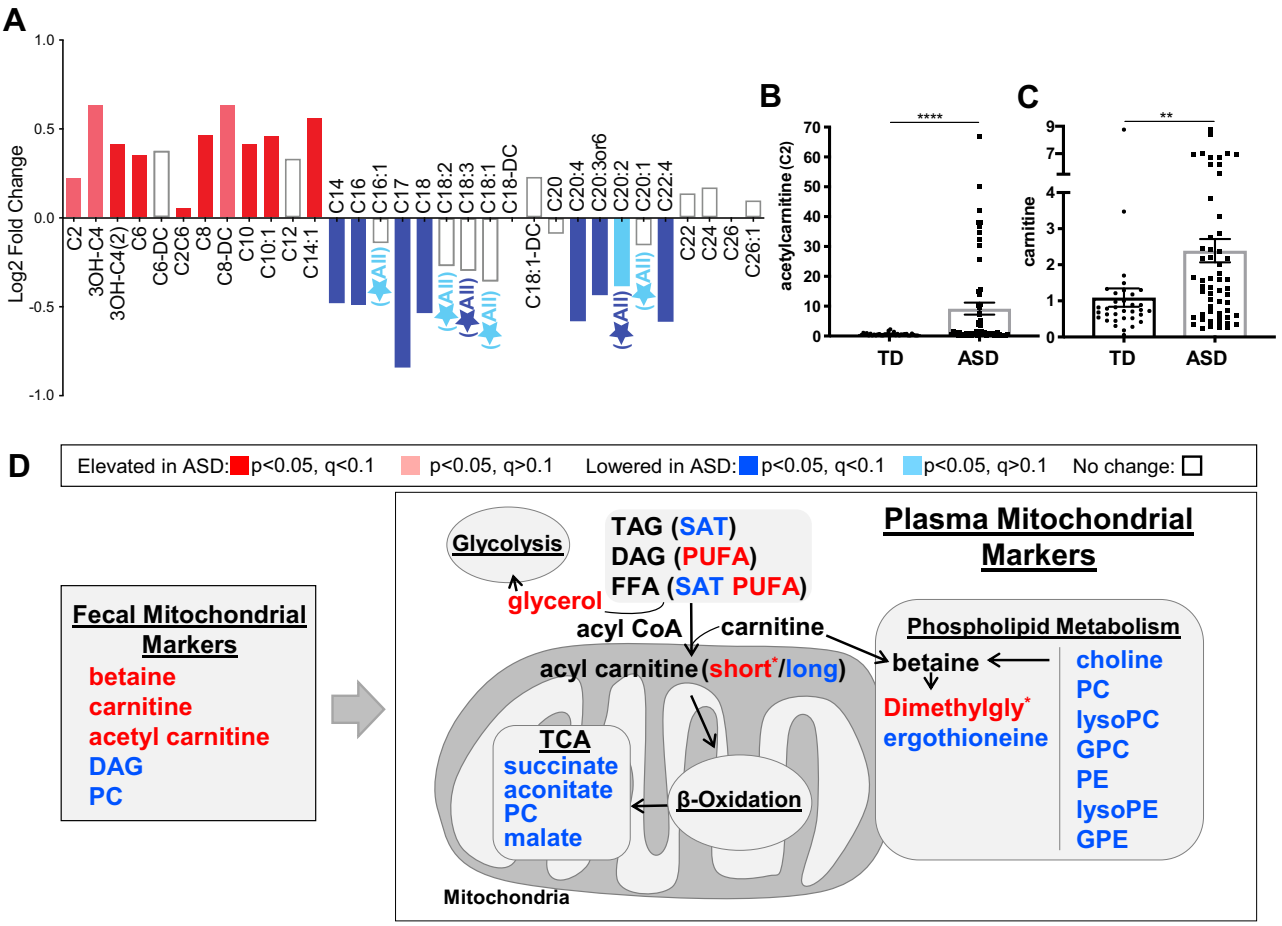



**Figure 3.** Autism spectrum disorder (ASD) abnormalities within cellular energy and oxidative stress metabolites. **(A)** $Log_2$ fold change of acylcarnitines in the plasma of ASD samples without gastrointestinal symptoms compared with typically developing (TD) control samples. Significance indicated by color according to legend below, determined by analysis of variance contrasts. Star indicates that a trend or significance was observed but only in the comparison between all samples. **(B, C)** Scaled intensity values indicating relative levels of acetylcarnitine (C2) and carnitine in ASD fecal samples compared with TD control samples (all samples). Data are represented as mean ± SEM. Asterisks indicate significance in analysis of variance contrasts (false discovery rate cutoff $q < .1$) performed on total metabolomics dataset (**$p < .01$, ****$p < .0001$). **(D)** Schematic of mitochondrial markers and other metabolites closely associated with cellular energy in plasma (within center box) and feces (boxed to left). *Significant only in ASD samples without gastrointestinal symptoms. Color of text indicates direction and significance of change according to legend above. DAG, diacylglycerol; FFA, free fatty acid; GPC, glycerophosphocholine; GPE, glycerophosphoethanolamine; GPG, glycerophosphoglycerol; GPI, glycerophosphoinositol; GPS, glycerophosphoserine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PUFA, polyunsaturated fatty acid; SAT, saturated fatty acid; TAG, triacylglycerol.

## Steroid Hormone Levels Are Elevated in ASD

Multiple human ASD studies have examined the levels of specific steroid metabolites within androgenic, pregnenolone, and progesterone metabolism, with some finding aberrant levels, positive correlations with ASD severity, and behavioral improvement following treatments that lower levels of certain hormones (49,65,74–81). In contrast, in a recent clinical trial of children with ASD given an antioxidant treatment, levels of pregnenolones and androgens increased and correlated with improved behavior (54). In our dataset, we found increases of many detected metabolites within the pregnenolone and androgen pathways (Figures 1C, 4A; Table S1 in Supplement 2). This is an indicator that the physiological pathways associated with the downstream metabolism of cholesterol are significantly altered between ASD and TD populations. There does not appear to be a global change in steroid metabolism, as most primary bile acid and sterol metabolites were unaffected (Tables S1 and S4 in Supplement 2). We observed some elevation of these hormone levels independent of sex, which is notable considering the male bias in ASD, reflected in our primarily male sample set (7%–15% female) (Figure S6A, B in Supplement 1) (82). Because a cluster of our samples are from older individuals in the ASD group, and to account for age-dependent increases in androgens (Table S7 in Supplement 2), we stratified by age and still observed heightened androgenic and pregnenolone metabolite levels in ASD subpopulations (Figure S6C in Supplement 1). Taken together, these data indicate that steroidal hormone metabolism may be altered in the ASD population relative to TD samples and that these differences are not driven solely by sex or age differences in our cohort.

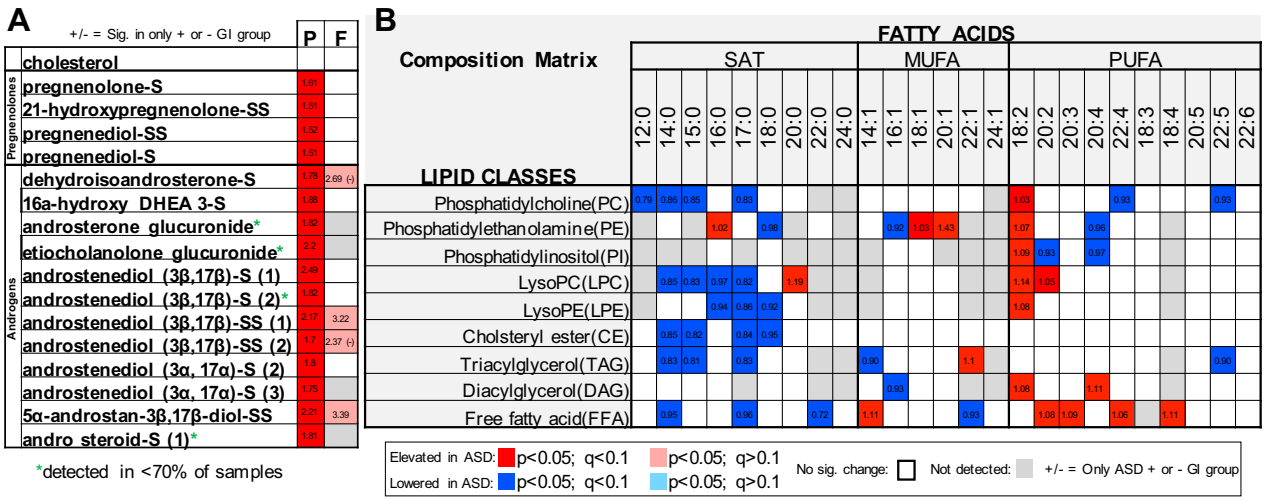



**Figure 4.** Steroid hormone levels are elevated in autism spectrum disorder (ASD), and other lipid metabolite levels differ in ASD. **(A)** Significant alterations to levels of all metabolites are detected in the pregnenolone, progestin, and androgen steroid pathways in plasma (P) and feces (F), with colors indicating significance and fold change direction according to legend at bottom right and numerical fold change in text within the box. **(B)** Complex lipid panel results for all ASD plasma samples compared with typically developing control samples with acyl chain length of lipids across the top, described by chain length and degree of unsaturation and categorized by saturated (SAT), monounsaturated (MUFA), and polyunsaturated (PUFA) fatty acids. Lipid classes are listed along the left. Direction of change and significance are indicated by the legend. Significance determined by analysis of variance contrasts; false discovery rate cutoff $q < .1$. DHEA, dehydroepiandrosterone; GI, gastrointestinal (symptoms); sig., significant.

## Lipid Metabolite Levels Differ in ASD

Lipids are crucial for energy storage, cellular membrane integrity, and cell signaling. They play a variety of roles in the central nervous system, and their dysregulation has been linked to ASD (11,13). Phospholipids have been measured at lower levels, while long-chain fatty acids are reportedly elevated, but polyunsaturated fatty acids (PUFAs) have been measured at both higher and lower levels, depending on the cohort (11,14,15). Here, we performed a comprehensive, quantitative metabolite analysis on complex lipids. The concentrations of 999 lipids were quantified, and 13.7% of these were significantly different in the ASD samples, with 4.6% increased and 9.1% decreased compared with TD samples. Many of these differentially abundant lipids included phospholipids, cholesterol esters, and glycerolipids (Figure S7A in Supplement 1). In general, shorter (14–18 chain length) saturated fatty acids were less abundant in ASD throughout the lipid classes (Figure 4C; Figure S7B in Supplement 1).

PUFA lipid levels were also different, with elevations in diacylglycerols and free fatty acids and an enrichment of the 18:2 (linolenic) chain length in most lipid classes (Figure 4C). Fecal samples generally trended in the opposite direction from plasma in PUFA lipids (Figure S7C in Supplement 1; Table S4 in Supplement 2). PUFA lipids containing linolenic acids are precursors to other PUFAs (arachidonic and docosahexaenoic acids) important for brain development, function, and structural integrity (14). Future studies are needed to determine if these changes in lipid levels contribute to ASD.

Considering the prevalence of intestinal issues in ASD, GI-dependent metabolite analysis between individuals may provide useful insights. Accordingly, we found a total of 87 and 24 metabolites in plasma and feces, respectively, that were potentially differential within ASD+GI compared with ASD−GI individuals by analysis of variance contrasts, but none passed the false discovery rate cutoff (Figure S5A, B in Supplement 1). At the pathway level, we found enrichments in free fatty acids between ASD+GI and ASD−GI plasma samples (Figure S5C in Supplement 1), but none in fecal samples (Table S4 in Supplement 2). In plasma, free fatty acids of multiple chain lengths trended lower in ASD+GI compared with ASD−GI samples (Figure S5D in Supplement 1). Some fatty acids, such as PUFAs, are anti-inflammatory, and their lower levels may contribute to GI dysfunction directly (14). We did not observe significant correlations between lipid (or other) metabolite patterns and GI symptoms or disease, including diarrhea, constipation, or irritable bowel syndrome. While these results are interesting, the implications of correlating an altered metabolome and GI symptoms in ASD remain to be determined.

## Differential Phenolic Xenobiotic Metabolite Levels in ASD

Phenolic metabolites, a diverse structural class of thousands of molecules containing a phenol moiety, come from dietary ingredients or biotransformation of aromatic amino acids by the gut microbiota. In their free phenolic forms, they can be readily absorbed through intestinal tissues; however, microbial modification of these molecules can significantly alter their absorption, bioavailability, and bioactivity (83), leading to various benefits or harm to the host (83,84). In fact, altered levels of phenolics have been highlighted in many ASD metabolomic studies (20,22–28,30,33,34,54,71,85,86). However, a consensus of these metabolite changes in ASD has yet

A

| Sig. Altered Metabolite | P | F |
|---|---|---|
| 4-ethylphenyl sulfate | 6.89 | |
| 3-hydroxybenzoate | | 0.7 |
| 2-ethylphenyl sulfate | 0.72 | |
| phenol sulfate | 0.84 | |
| 3-(4-hydroxyphenyl)lactate | 0.9 | |
| 4-allylphenol sulfate | 0.72 | |
| salicyluric glucuronide | 0.88 | |
| 2-aminophenol sulfate | 0.67 | |
| 4-methylbenzenesulfonate* | 59.6 (-) | |
| genistein sulfate* | 5.44 (+) | |
| equol glucuronide* | 2.0 (+) | |
| equol sulfate* | 2.04 (+) | |
| daidzein sulfate (2)* | 3.41 | |
| salicylate | 0.89 (-) | 0.49 |
| 3-hydroxyhippurate | 0.77 (-) | |
| caffeic acid sulfate | 0.62 (-) | |
| hydroquinone sulfate | 0.72 (-) | |
| 4-hydroxyhippurate | 0.86 (-) | |
| isoeugenol sulfate* | 0.46 (+) | |
| 2-hydroxyhippurate | 0.89 | |
| o-cresol sulfate* | 0.58 | |
| vanillactate* | 0.82 | |
| vanillic alcohol sulfate | 0.63 | |
| 4-hydroxycoumarin | 0.86 | |
| m-tyramine* | | 5.4 |
| 4-hydroxybenzoate | | 0.51 (+) |
| 4-methylcatechol sulfate* | | 0.61 |
| p-cresol sulfate | | 0.48 (+) |
| gentisate | | 0.75 |
| 3-hydroxyphenylacetate | | 0.49 |
| dihydroferulic acid | | 0.5 |
| sinapate | | 0.34 |

ASD/TD
Elevated: p<0.05; q<0.1 p<0.05; q>0.1
Lowered: p<0.05; q<0.1 p<0.05; q>0.1
No sig. change: Not detected:
+/- = Only ASD + or - GI group
*detected in <70% of samples

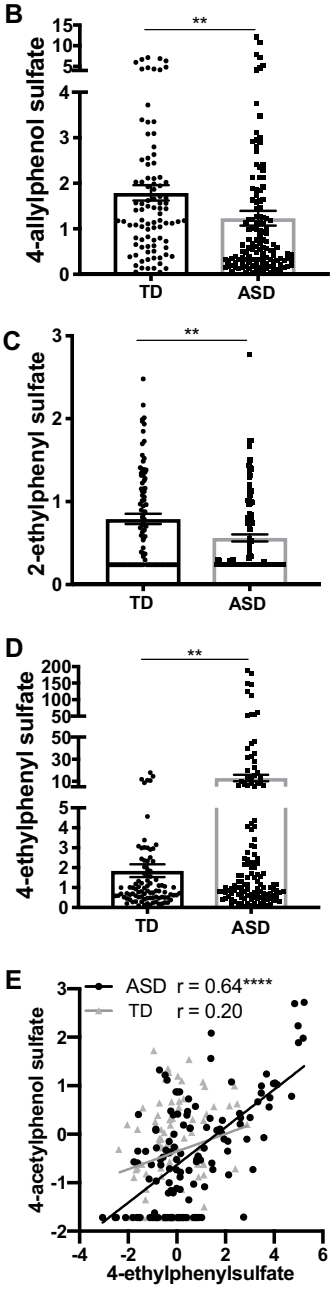



**Figure 5.** Differential phenolic xenobiotic metabolite levels in autism spectrum disorder (ASD). **(A)** Phenolic metabolites belonging to the benzoate, tyrosine, and food component/plant pathways that are significantly different between ASD vs. typically developing (TD) groups in plasma (P) and feces (F). Directionality and significance defined in legend, and numerical fold change in text within the box. **(B)** Scaled intensity values indicating relative levels of 4-allylphenol sulfate in plasma (all samples). **(C)** Scaled intensity values indicating relative levels of 2-ethylphenyl sulfate in plasma (all samples). **(D)** Scaled intensity values indicating relative levels of 4-ethylphenyl sulfate in plasma (all samples). **(E)** Spearman correlation between plasma 4-ethylphenyl sulfate and 4-acetylphenol sulfate, $\log_2$ scale. Data in panels **(B–D)** are represented as mean ± SEM. Asterisks indicate significance in analysis of variance contrasts with a false discovery rate cutoff $q < .1$ performed on total metabolomics dataset (**$p < .01$, ****$p < .0001$). GI, gastrointestinal (symptoms); sig., significantly.

to be reached, and most studies have measured only a subset of these metabolites.

We observed altered or trending levels of phenolic metabolites belonging to several interrelated pathways, including tyrosine, benzoate, and food component/plant metabolites (Figure 5A). Some share structural similarity to para-cresol sulfate, a toxic molecule elevated in the urine of young children with ASD (22,24). These include, among others, 4-EPS, 2-ethylphenylsulfate, cresol derivatives, 4-allylphenyl sulfate, and 4-methylbenzenesulfonate; the last-mentioned was elevated a remarkable 60-fold in the small subset of ASD samples in which it was detected (Figure 5A–D; Table S1 in Supplement 2). Changes in the levels of these phenolic molecules have also been observed in animal models of ASD (52,87). Previously, 4-EPS was observed with a 46-fold elevation in the maternal immune activation mouse model, and administration of synthetic 4-EPS to wild-type mice was sufficient to induce an anxiety-like phenotype (52). Here, in ASD plasma samples, 4-EPS levels were increased 6.9-fold (Figure 5D). This was largely driven by a considerable subpopulation of individuals with ASD with high

| | Metabolite(s) | Core Findings |
|---|---|---|
| Lipids | Steroid hormones | Pathways enriched; pregnenolone and androgen hormone metabolite levels elevated |
| | PUFAs | Elevated levels in DAG, FFA, and many with chain length 18:2 |
| | Saturated FAs | Pathways enriched; broad decrease in levels |
| | Free FAs | Trending decreased levels in ASD+GI |
| Markers of Mit. Function | Acyl-carnitines | Pathways enriched; shorter chain moieties elevated, longer chain moieties decreased; acetyl- and free carnitine elevated in feces |
| | Phospholipid metabolism | Pathway enriched in feces |
| | TCA cycle | Lower levels of succinate, aconitate, malate levels |
| | Pathways rel. to oxidative stress (Cys, Met., SAM; glutathione; gg-AA metabolites) | Significant correlations with ADOS SS |
| Xenobiotic and Phenolic Metabolites | Phenolic structure mets.: 4-ethylphenyl sulfate (4EPS), genistein-S, equol mets., daidzein sulfate, 4-methylbenzene sulfonate | Elevated to various degrees in ASD plasma samples |
| | Phenolic structure mets.: hippurate and benzoic acid mets., some tyrosine mets., salicylic mets. | Decreased to various degrees in ASD plasma samples |
| | Phenolic structure mets.: equol-S, 3-phenylpropionate, hippurate, 4-hydroxyhippurate, caffeic acid-S, phenol-S, daidzein-S, genistein-S | Display shifted levels in mouse recipients of human ASD fecal samples compared to TD fecal samples |
| Potential Biomarkers | Acyl-carnitines and lipids | Rank in the top 30 most discriminatory metabolites, assessed by Random Forest analysis |
| | Metabolites known to be influenced by the gut microbiota | Rank in the top 30 most discriminatory metabolites, assessed by Random Forest analysis |
| | Indolelactate, margaroylcarnitine | Most discriminatory plasma metabolites |
| | Nicotinamide, 9-HOTrE | Most discriminatory fecal metabolites |

**Figure 6.** Summary chart of core findings. Categorized into metabolites of lipid, mitochondrial function marker, and xenobiotic and phenolic pathways as well as potential biomarkers, the metabolites of most interest (left) and observations made from the data (right) are summarized. ADOS, Autism Diagnostic Observation Schedule; ASD, autism spectrum disorder; Cys, cysteine; DAG, diacylglycerol; FAs, fatty acids; FFA, free fatty acid; gg-AA, gamma-glutamyl amino acids; GI, gastrointestinal (symptoms); Met., methionine; mets., metabolites; Mit., mitochondrial; PUFA, polyunsaturated fatty acid; SAM, *S*-adenosyl-L-methionine; SS, severity score; TCA, tricarboxylic acid; TD, typically developing.

levels of 4-EPS, who also show higher levels of variation in related phenolic metabolites (Figure S8A in Supplement 1). In many cases, phenolic metabolite levels correlate with 4-EPS in ASD samples, including 4-acetylphenyl sulfate, a derivative of 4-EPS, and others (Figure 5E; Figure S8B, S8C in Supplement 1). These observed alterations, combined with mounting evidence from previous studies, suggest that phenolic metabolites may be involved in ASD.

## DISCUSSION

Changes in the metabolome have been linked to a number of neurodevelopmental and neurodegenerative disorders (88,89). The current study includes a comprehensive profiling of the metabolites from 231 plasma and 97 matched fecal samples from individuals with ASD and TD who underwent extensive behavioral testing. We observed that specific individual metabolites and metabolic pathways in lipid, amino acid, and xenobiotic metabolism were altered between ASD and TD groups and that many of these same molecules and pathways correlated to severity of ASD behavioral scores. The core findings are summarized in Figure 6. Various steroid hormone metabolite levels were elevated in ASD samples. PUFA levels and short-chain acylcarnitines were generally elevated in ASD plasma samples, while saturated fatty acid levels and long-chain acylcarnitines were decreased, contributing to a general picture of dysregulated cellular energy and oxidative state, with potential connections to mitochondrial dysfunction in ASD. Some of the lipid and xenobiotic metabolites also showed interesting changes within the ASD group when stratified by the presence of GI symptoms. Finally, many phenolic metabolites derived largely from host and bacterial metabolism of amino acids, plant polyphenols, and other food components were detected at differential levels in plasma and feces between groups.

ASD is diagnosed by behavioral tests, with extensive heterogeneity of symptoms, severity, and etiology between individuals and little consensus on molecular mechanisms. This enigmatic spectrum has a strong but complex genetic basis, with hundreds of reported risk genes (90). The contributions of risk alleles to behavior is a vast and active area of research. In addition to genetics, understanding of altered metabolite levels in blood, feces, and brains of individuals with ASD may provide a glimpse into physiological aspects of the disorder and hold the potential to advance diagnosis and/or stratification of subpopulations of ASD. In fact, our results broadly support the notion of multiple etiologies or endophenotypes within the ASD population.

Interestingly, multiple mouse models support the notion that gut metabolites are associated with brain development and function (50,91,92). Some metabolites are closely linked with neurological disorders, either to positive or to negative outcomes (93,94). Several examples have been reported of gut metabolites entering circulation and directly affecting the brain, as well as cases where metabolites stimulate pathways in the gut, immune, or autonomic nervous system and exert changes to the brain and to behavior (95–98). Comprehensive metabolic profiling in humans and animal models provides insight into the molecular status of disease and how genetic factors and environmental influences (dietary, microbial, and chemical) interact. Deeper analysis of our dataset along with additional studies, with future empirical studies to validate the relevance of our observations, could illuminate aspects of ASD pathophysiology. The intriguing correlations between ASD behaviors, altered levels of fecal and plasma metabolites, and GI

symptoms contribute to the concept that ASD may be viewed as a whole-body condition and argue for increased investigation into peripheral aspects of disease that may lead to advances in diagnosis and improved stratification of ASD populations.

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## ARTICLE INFORMATION

From the Division of Biology and Biological Engineering (BDN, MDA, SKM), California Institute of Technology, Pasadena; Department of Medical Microbiology and Immunology (DRR, PA), University of California, Davis, Davis; MIND Institute (DRR, PA), University of California, Davis, Sacramento, California; Division of Pediatric Gastroenterology and Nutrition (GS, AF), Mucosal Immunology and Biology Research Center, Massachusetts General Hospital for Children, Boston; and Axial Biotherapeutics (GMP, MCC, ASC, DHD), Waltham, Massachusetts.

Address correspondence to Sarkis K. Mazmanian, Ph.D., at sarkis@caltech.edu, or Brittany D. Needham, Ph.D., at bneedham@caltech.edu.

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