Title

Reanalysis of metabolomics data reveals that microbiota transfer therapy modulates important fecal and plasma metabolite profiles in children with autism spectrum disorders

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Running Title: Reanalysis of metabolomics data for microbiota transfer therapy in Autism

Abstract:

While Autism Spectrum Disorder (ASD) is diagnosed through behavioral symptoms and psychometric evaluations, it has also been associated with distinct metabolomic patterns. A previous clinical trial of Microbiome Transfer Therapy (MTT) in children with ASD and gastrointestinal (GI) issues revealed significant differences in plasma metabolomics between children with ASD and their typically developing (TD) counterparts, which diminished after MTT. The objective of this study was to reanalyze the plasma and fecal samples using updated metabolomics libraries at Metabolon, applying a comprehensive panel of statistical methods. This approach aimed to provide deeper insights into ASD-related metabolic differences and the impact of MTT. The reanalysis identified more statistically significant metabolites and highlighted specific metabolites whose relative intensities differed between the ASD and TD groups, as well as metabolites with significant changes in intensity post-MTT. Several plasma metabolites, including sarcosine, iminodiacetate, caproate, and caprylate, initially showed significant differences between the ASD and TD groups but shifted to resemble TD levels after MTT treatment. In fecal samples, p-cresol sulfate and sphingolipids emerged as metabolites with altered intensities following MTT treatment. Multivariate Fisher's Discriminant Analysis (FDA) with leave-one-out cross-validation revealed that a set of metabolites—including p-cresol sulfate, hydroxyproline, and caprylate—could robustly classify the ASD and TD cohort's pre-treatment. However, after treatment, the same FDA model could not distinguish the two groups, as the FDA scores became similar to those of the TD cohort.

Our findings enhance the understanding of ASD-associated metabolic changes and highlight the potential of MTT to influence these profiles. This underscores the importance of reanalysis using updated databases and robust statistical methods for comparative analysis. Further studies with larger cohorts and placebo-controlled trials are necessary to validate these results and explore the biochemical pathways involved, paving the way for personalized treatment approaches.

Introduction

In the past decade, the prevalence of autism spectrum disorder (ASD) in the United States has significantly increased, with 1 in 36 children now diagnosed (CDC, 2023). ASD individuals experience social communication and behavioral impairments (Adams et al., 2011; Lord et al., 2018), and often have persistent gastrointestinal (GI) issues, such as constipation and diarrhea (Adams et al., 2011; Hung et al., 2023). These co-occurring conditions correlate with increased ASD severity (Adams et al., 2011), prompting research into the gut-brain axis, gut microbiome, and ASD's behavioral severity. Studies reveal significant differences in gut microbiome compositions between ASD individuals and typically developing (TD) children, including higher levels of *Clostridia* species and lower levels of beneficial microbes like *Bifidobacterium* (Kang et al., 2019; Morton et al., 2023).

The complex interaction among microbes, influenced by their metabolites, has been hypothesized to affect GI and behavioral symptoms in ASD (Adams et al., 2011; Krajmalnik-Brown et al., 2014; Kang et al., 2020; Needham et al., 2021; Morton et al., 2023; Hung et al., 2023; Zheng et al., 2023). Differences in metabolite profiles in fecal, urine, and blood samples have been reported in ASD individuals compared to TD peers (Gabriele et al., 2014; Kang et al., 2018; 2020; Needham et al., 2021; Zheng et al., 2021). For example, elevated levels of fecal short-chain fatty acids and blood glutamate, along with increased blood gamma-aminobutyric acid (GABA), have been observed in people with ASD (Shinohe et al., 2006; Kang et al., 2018; El-Ansary et al., 2014; Khalifa et al., 2019). GABA and glutamate, which are critical for cognition and mood regulation, show an altered balance in ASD individuals, suggesting an underlying neuroinflammation (El-Ansary et al., 2014). Additionally, other gut-derived metabolites, such as p-cresol and p-cresol sulfate, are found at higher levels in ASD individuals' urine, feces, or serum (Kang et al., 2018; De Angelis et al., 2013; Gabriele et al., 2014).

Given the microbiome-gut-ASD interconnection, Microbiota Transfer Therapy (MTT) has been explored for its potential benefits in ASD (Kang et al., 2017). MTT has shown promise in addressing GI issues and reducing behavioral severity in ASD children. An open-label study by Kang et al. reported an 80% reduction in GI symptoms and a 24% decrease in core ASD symptoms post-MTT, with sustained improvements over two years (Kang et al., 2017, 2019).

Increases in beneficial microbes such as *Bifidobacterium*, *Prevotella*, and *Desulfovibrio* were also noted.

Building on these findings, untargeted metabolomics of fecal and plasma samples from the same individuals in the MTT study identified several plasma metabolites significantly lower in the ASD group at baseline, including nicotinamide riboside, IMP, iminodiacetate, methylsuccinate, galactonate, valylglycine, sarcosine, and leucylglycine, while caprylate and heptanoate were higher (Kang et al., 2020). Though no fecal metabolite showed significant baseline differences, *p*-cresol sulfate levels were non-significantly higher in ASD children and decreased after MTT (Kang et al., 2020). Multivariate analysis highlighted significant metabolite changes post-MTT, with improvements in sarcosine and inosine 5'-monophosphate in plasma (Adams et al., 2019) and a model of indole, imidazole, propionate, adenosine, and hydroxyproline achieving 96% accuracy in distinguishing ASD from TD children (Qureshi et al., 2020).

In this study we reanalyzed the metabolomics data collected for the Kang et al. 2017 and 2019 studies. We extend upon the findings reported by Kang et al. (2020) by expanding the scope of metabolomics data analyzed through Metabolon updated databases and robust statistical methods. Utilizing more up-to-date algorithms and libraries for metabolite detection, we offer a comprehensive comparison of metabolite profiles, encompassing nucleotides, neurotransmitters, amino acids, xenobiotics, and lipids derived from human plasma and fecal samples collected from a carefully matched cohort of individuals with ASD and typically developing children. Previously, data was analyzed using univariate tests and principal-component analysis (PCA) and correlation analysis for selective metabolites. In this reanalysis, we not only replicated the prior analysis techniques, but we also explored other methods such as contrasting the area under the receiver operating characteristic curve (AUROC) between groups, random forest, and Fisher discriminant analysis (FDA) to revalidate the findings (Fig. 1). The results reveal significant differences in metabolite levels between TD and ASD baseline groups in plasma and feces, which were previously unidentified, and important changes shifting the ASD metabolon towards TD after MTT.

Methods

Study design, sample collection and data processing

This study extends the work of Kang et al. (2020) by performing a more thorough reanalysis of plasma and fecal metabolomics data collected before and after MTT in children with ASD. The original protocol was approved by the U.S. Food and Drug Administration (FDA) (investigational new drug number 15886) and the Institutional Review Board of Arizona State University (ASU IRB protocols #00001053 and #00004890). The trial was registered at ClinicalTrials.gov (NCT02504554), as described in Kang et al. (2017, 2020). Study participants, consent, microbiota transfer therapy (MTT), and sample collections were described in detail in our previous studies (Kang et al., 2017; 2020). In total, 20 typically developing (TD) children and eighteen children with ASD were recruited, in the age range of 7-16 years old. Children with ASD participated in an open-label clinical trial including vancomycin for 2 weeks, one-day bowel cleanse with MoviPrep, followed by 7-8 weeks of MTT and Prilosec (a stomach acid suppressant), and a follow-up at 8 weeks post-treatment (i.e., 18 weeks from Day 0) (Kang et al., 2017).

For metabolomic analysis, we collected plasma samples at ASD baseline, week 3 (after the vancomycin and bowel cleanse and just before the first dose of microbiota), and week 10 (the end of MTT treatment) (Fig. 1). We had one missing plasma sample in week 3. Fecal samples were collected at baseline, week 3 (right after an initial dose of microbiota), week 10 and week 18 (8 weeks after the end of MTT). For TD children, we collected plasma and feces only at baseline as they were not treated. All samples were frozen immediately after collection, shipped in dry ice to ASU, and stored at -80°C. Demographic, medical data and diet history was recorded, as described by Kang *et al*., 2017, 2020. Aliquoted frozen samples were analyzed by Metabolon Inc. (Durham, NC, USA) using ultrahigh-performance liquid chromatography-tandem mass spectroscopy (UHPLC-MS/MS) instruments for untargeted metabolomics.

Raw data underwent imputation and z-score normalization according to the methods described by Kang et al., 2020. This reanalysis leverages upgrades in peak identification algorithms, software, and libraries available to Metabolon Inc. (https://www.metabolon.com/). Using these extended approaches new data were generated, resulting in modified abundances of metabolites (peak area/intensity) and the identification of additional metabolites within the samples.

Univariate analysis

Initially, univariate hypothesis testing was employed to compare metabolites between groups. Depending on the data distribution and type of relationship of the two groups, the most appropriate statistical test was selected. Mann-Whitney was utilized for comparing non-parametric data with similar distributions between the TD and ASD samples, while a t-test was used in alternative cases. When comparing ASD groups at various time points a Wilcoxon sign-rank test was used for non-parametric distributions and a paired t-test was used for parametric distributions. False discovery rates (FDR) were determined using the leave-one-out (LOO) approach. This technique applies the same hypothesis testing approach to every combination of a dataset with one sample excluded. Then, the proportion of significant findings is determined to evaluate the likelihood of false discovery. Subsequently, findings with $p < 0.05$ and FDR <0.05 were considered statistically significant.

Area Under the Receiver Operating Characteristic Curve (*AUROC)*

The area under the receiver operating characteristic curve (AUROC) was used to assess the degree to which metabolite measurements were discernible between pre-treatment and post-treatment time points. For a binary classification task, the AUROC is determined by plotting the true positive rate (TPR) against the false positive rate (FPR) of a model across varying threshold values. The area under the TPR/FNR plot corresponds to the AUROC and can serve as a metric to quantify the degree to which different groups are separable. The AUROC value ranges from 0 to 1, where a value of 0.5 indicates no discrimination, a value of 1 represents perfect discrimination, and a value close to 0 suggests that the model is performing worse than random, potentially predicting the opposite of the true outcome.

The AUROC was calculated by treating the measurements of each metabolite individually as the classifier input, which was subject to a threshold value. AUROC was determined for each fecal and plasma metabolite, with baseline measurements being compared against all other time points. The measurements collected from the ASD group were also compared against the TD measurements, both in their entirety and on a timepoint basis. By assessing all the AUROC values generated for each metabolite between baseline, week3, and week10 vs TD across, the average fecal and plasma AUROC values for each group were determined.

Fisher's Discriminant Analysis (FDA)

FDA is a dimensionality reduction technique that aims to distinguish between different groups, and can be used in creating multivariate models for classification tasks. FDA endeavors to find a transformation of the original data that accentuates the differences between distinct groups while reducing the variations within each group. This method projects multidimensional data into a lower dimensional space in such a way as that dissimilar groups are maximally separated, while members of the same group remain closely grouped together. By doing so, FDA facilitates the creation of more discriminative representations (Huberty, 1975).

Metabolites that had been deemed to be statistically significant in both plasma and feces were used to develop an FDA model. To mitigate the risk of overfitting, feature selection was limited to five variables, as literature suggests this approach optimally suits small sample sizes with similar data structure properties in ASD prediction. This decision is also supported by the success of prior fecal and plasma model panels achieving the upper bounds of their predictive accuracy with this number of features (Howsmon et al., 2017; Vargason et al., 2019; Qureshi et al., 2020). An exhaustive search of all potential FDA model combinations of five metabolites was determined, using only the ASD baseline and TD groups as training data. For all possible combinations of five metabolites, the AUROC was evaluated to determine whether to conduct further testing. Each metabolite combination model that achieved an AUROC greater than 0.97 was subjected to a leave-one-out cross-validation (LOO-CV) paradigm. The top-performing model as quantified by CV accuracy was then used to predict the classification of the MTT week3 and week10 groups.

Random Forest Analysis

Random forest (RF) is defined as an ensemble learning method that can be used for classification. An RF approach to classification operates by aggregating the collective performance of multiple decision trees and using the plurality of outcomes to predict the group label of a sample. This algorithm is constructed by first setting aside one-third of the training data to utilize as testing data and utilized for cross-validation purposes. Subsequently, a user-specified number of decision trees are constructed. The performance of the algorithm is then quantified based on the classification accuracy on the randomly selected test set. A random forest model was fit using all significant metabolomic measurements in order to evaluate the mean

decrease in accuracy for the exclusion of each variable. This metric is commonly utilized to provide a perspective on feature importance, which can be used to more efficiently identify variables better suited for distinguishing between labeled categories (Altman et al., 2017).

Correlation Coefficient

The Pearson‘s correlation coefficient is defined by the strength and direction of the linear relationship between two continuous variables. The Pearson’s correlation coefficient was determined between all metabolite, pathway and taxa pairs. The significance of the correlation coefficients was evaluated using hypothesis testing with a significance level set at $\alpha = 0.05$. The robustness of significant findings was cross-validated using a LOO approach. Employing this strategy, the significance of the correlation coefficient was determined for all possible combinations of the input measurement with a single sample removed. If a false discovery rate (FDR) of less than 0.05 was determined, the finding was deemed to be significant.

All univariate analysis, area under the receiver operating characteristic (AUROC), random forest analysis and plots were made with Python (v3.8.5) in Jupyter Notebook (v6.1.4) using numpy (v1.19.2), matplotlib (v3.3.2), seaborn (v0.11.0), pandas (v1.1.3) and scipy (v1.5.2). PCA plot was made using MetaboAnalyst v6.0 (https://www.metaboanalyst.ca/). BioRender (Biorender, 2022, Licensed, https://biorender.com) and Inkscape (v1.1) were used to create or edit the figures.

Results

In the previous study (Kang et al., 2020), 621 plasma and 666 fecal metabolites were identified in the collected samples. However, through reprocessing and reanalysis of the metabolomics data using new updated reference library, a greater number of biochemical compounds were identified from the same samples. We identified a total of 844 plasma and 825 fecal metabolites, and most of the newly identified metabolites in plasma and feces were lipids, amino acids, or xenobiotics (Fig. 2).

Metabolite profiles changed after MTT in ASD children

Univariate analyses showed that certain metabolites that were initially different between ASD and TD groups at baseline, shifted following MTT. These metabolites relative intensity changed post-treatment, and some of these intensities became similar to those in TD in plasma (Fig. 3A, C) and fecal samples (Fig. 3B, Supplementary S1). In plasma, out of 844 metabolites, we observed 20 metabolites whose intensity was significantly different between the ASD and TD and that also changed after MTT (Fig. 3A). We grouped these plasma metabolites into two clusters; **1)** Cluster P-high (cluster of 14 plasma metabolites), which includes metabolites that had significantly higher intensity (adjusted $p<0.05$) at ASD baseline vs TD, but after MTT, the relative intensity of these metabolites decreased and became similar to TD (Fig. 3A). Figure 3A shows 12 plasma metabolites in this category, including: glycerophosphoethanolamine (GPEA), caprylate (8:0), and caproate (6:0). The other two plasma metabolites, taurine and adenosine-5'-monophosphate (AMP), were non-significantly higher ($p>0.05$) at baseline vs TD but significantly decreased after MTT (Fig. 3A). **2)** Cluster P-low (cluster of 6 plasma metabolites) which includes metabolites that had significantly lower intensity (adjusted $p<0.05$) at baseline vs TD, but after MTT, relative intensity of these metabolites significantly increased (adjusted $p<0.05$) and became similar to TD. Cluster P-low includes sarcosine, iminodiacetate, bilirubin (Z,Z), 2-methylserine, and indole propionate.

Similar to plasma, out of 825 fecal metabolites, we observed 22 metabolites that had significantly different intensities from TD or changed after MTT (Fig 3B, Supplementary Fig. S1). Fecal metabolites were also divided into two clusters; **1)** Cluster F-low (cluster of 3 fecal metabolites) where all metabolites had significantly lower intensity (adjusted $p<0.05$) at baseline vs. TD, and **2)** Cluster F-high (cluster of 19 fecal metabolites) where all metabolites had higher intensity (adjusted $p<0.05$) at baseline vs. TD. Cluster F-low (Fig. 3B-) shows 3 fecal metabolites pentadecanoate (15:0), myristate (14:0), and carboxyethyl-GABA whose intensity was significantly lower at baseline vs. TD, and only one metabolite pentadecanoate (15:0) whose intensity significantly increased (adjusted $p<0.05$) after week18 (Fig. 3B). In cluster F-high, 19 metabolites had significantly higher intensity (adjusted $p<0.05$) at baseline vs TD, here the intensity of 5 metabolites: *p*-cresol sulfate, throebromine, oxalate ethanedioate, dodecanedioate (C12), and Urolithin-A significantly decreased (adjusted $p<0.05$) at week3 vs baseline (Fig. 3B). The intensity of: N-palmitoyl-sphingosine (d18:1/16:0), *p*-cresol sulfate, eicosanoyl-sphingosine

(d20:1), hydroxyproline, theobromine, N-stearoyl-sphingosine (d18:1/18:0), N-oleoyl-sphingosine (d18:1/18:1), 3-methyl-2-oxobutyrate, dodecanedioate (C12), and D-urobilin significantly decreased after week18 compared to baseline. We did not observe any significant change for week10 vs. Baseline (Fig. 3B).

Analytics and Patterns of Differential Metabolomic Composition Appear Similar between Feces and Plasma

We utilized AUROC, PCA analysis and random forest analysis to categorize the differences in metabolomic composition across groups and timepoints. AUROC was calculated for all 20 plasma and 22 fecal metabolites that were significantly different between ASD baseline and TD. To examine how metabolites differed post-MTT, AUROC was recalculated at all time points between the ASD and TD cohort. The average AUROC for all plasma metabolites was 0.73 comparing baseline vs TD, and baseline vs after week3, and week10 AUROC's were 0.70 and 0.62 respectively (Fig. 4A). When we compared the MTT group with TD, after week3 and week10, we observed that the plasma profile became more similar to TD with AUROC 0.56 for week3 vs TD and 0.59 for week10 vs TD (Fig. 4A). A similar trend was also observed for fecal metabolites, which were initially observed with an AUROC of 0.74 for baseline vs TD. After MTT, fecal metabolite profiles became more similar to the TD group, with AUROC values of 0.58 for week3 vs TD and 0.61 for week10 vs TD (Fig. 4B). Overall, the observed shift in AUROC values for plasma and fecal metabolites indicates that after MTT, the metabolic composition in plasma and fecal samples from children with ASD became more similar to their TD counterparts.

PCA analysis showed that relative intensity of the 20 significant plasma metabolites at baseline were distinct from TD, but after MTT, became much more similar to TD (Fig. 4C). Fecal samples exhibited greater variance in the typically developing group compared to their ASD counterparts in the first two principal components, and this pattern was also observed in the post treatment ASD group (Supplementary Fig. S2). Random forest complimented the assessment of each biochemical compounds' relative utility in distinguishing between cohorts. Using the full list of metabolites, it was possible to achieve accuracies greater than 0.95. The mean decrease in accuracy for the ASD status prediction model indicated that both fecal and plasma metabolites had a similar influence on model performance (Supplementary Fig. S3). The

top 10 features contributing to mean decrease in accuracy were split evenly between the plasma and fecal metabolites, potentially offering complementary insights into discerning ASD and TD samples. (Supplementary Fig. S4).

MTT changed metabolites abundance in ASD towards TD

We selected four plasma metabolites based on biological impact in ASD individuals and that were significantly changed/different between groups. A lysine derivative sarcosine and iminodiacetate (IDA) were significantly lower (adjusted $p<0.05$) at baseline vs. TD. However, after week3 (after vancomycin) and week10 (MTT) the relative intensity was observed to significantly increase (adjusted $p<0.05$) and became more similar to TD (Fig. 3A, 5A-B). To quantify the changes, we calculated the AUROC, and sarcosine's intensity showed 0.82 AUROC at baseline vs TD, also at week3 (AUROC=0.73) and week10 (AUROC=0.85) vs. baseline were distinct (Fig. 6A). Notably, the AUROC at week3 and week10 compared to TD were 0.56 and 0.52, respectively (Fig. 6A), which indicate that after MTT, sarcosine's relative intensity became similar to TD. Similar findings were observed for iminodiacetate (Fig. 6B).

In contrast, we observed two medium chain fatty acids in plasma, caproate (6:0) and caprylate (8:0), that were significantly higher at baseline vs TD, but after week3 and week10 (MTT) significantly decreased (adjusted $p<0.05$) and their abundances became similar to TD (Fig. 5C-D). Caproate and caprylate AUROC's were 0.78 for baseline vs TD, which showed further differences comparing baseline to week3 (AUROC caproate=0.83, caprylate=0.79) and week10 (AUROC caproate=0.88, caprylate=0.90) vs. baseline, but the AUROC at week3 and week10 vs. TD was between 0.51-0.65 (Fig. 6C-D), which also indicates that after MTT, the relative intensity of caproate and caprylate became similar to TD.

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TD was between 0.51-0.65 (Fig. 6C-D), which also indicates that after MTT, the relative intensity of caproate and caprylate became similar to TD.

In feces, phenolic compound *p*-cresol sulfate, theobromine, and three sphingolipids; N-palmitoyl-sphingosine (d18:1/16:0), N-stearoyl-sphingosine (d18:1/18:0), and N-oleoyl-sphingosine (d18:1/18:1) were significantly higher at baseline vs. TD but after week3, week10 (MTT) and week18 (after 8 weeks of MTT) significantly decreased vs. baseline and became similar to TD (Fig 3B, Fig. 7A-D). AUROC calculation showed that baseline vs. TD had 0.76 for *p*-cresol sulfate, after baseline vs. week3 AUROC=0.80, vs week10 AUROC=0.66, vs week18 AUROC=0.75, which indicates after MTT, *p*-cresol sulfate profile changed in children with ASD (Fig. 8A). Interestingly, *p*-cresol sulfate AUROC for TD vs. week3; TD vs. week10; TD vs. week18 were in the range 0.55-0.60 (Fig. 8A), indicating that after MTT, *p*-cresol sulfate abundance became similar to TD. Similarly, theobromine, N-palmitoyl-sphingosine (d18:1/16:0), N-stearoyl-sphingosine (d18:1/18:0), and N-oleoyl-sphingosine (d18:1/18:1) had higher AUROC after MTT vs. baseline and lower AUROC values after MTT vs. TD, indicating important changes from baseline and greater similarity to TD measurements (Fig. 8B-D). Overall, AUROC validate the findings from univariate analysis and showed the differences between the groups for plasma and fecal metabolites.

FDA models revealed five key metabolites that notably changed after MTT and became similar to TD

Univariate hypothesis testing identified 20 plasma metabolites and 22 fecal metabolites whose intensities significantly differed between TD and ASD cohorts at baseline or changed after microbiota transfer therapy (MTT) (Fig. 3). Both sets of metabolites were used to separately construct multivariate statistical models *via* FDA using all possible combinations of 3, 4, and 5 variables to distinguish between ASD and TD cohorts. The top-performing FDA model made up exclusively of plasma metabolites included, 2-palmitoyl-GPC (16:0), caprylate (8:0), laurate (12:0), bilirubin (Z,Z), and indolepropionate achieving an AUROC of 0.99 and a leave-one-out cross-validated (LOO-CV) accuracy of 95%. Similarly, the plasma metabolite data was used to develop an FDA model, with significant features selected through the same univariate approach.

The optimal plasma model incorporated *p*-cresol sulfate, hydroxyproline, theobromine, N-oleoyl-sphingosine (d18:1/18:1), and 1-methylhistidine.

Utilizing the complete set of 44 significantly differing metabolites, all possible 5-variable FDA models were also constructed. Through LOO-CV, a model consisting of *p*-cresol sulfate (feces), hydroxyproline (feces), caprylate (plasma), indolepropionate (plasma), and bilirubin (Z,Z) (plasma) was identified as the most robust, with a cross-validation accuracy of 95% (Fig. 4D). This model exhibited a type I error rate of 3% and a type II error rate of 0%. Notably, the performance of all multivariate models for classifying between ASD and TD status significantly declined after MTT, as did the measurements taken at the two-year follow-up. The metabolite values in the model showed significant convergence towards the TD group, with the difference between the median of the five metabolites relative intensity values decreasing by 88% between week 0 and week18, compared to the TD group.

Fecal p-cresol and p-cresol sulfate are positively correlated

We conducted correlation analyses to examine the relationship between certain fecal and plasma metabolites. Fecal *p*-cresol and fecal *p*-cresol sulfate were significantly positively correlated ($p<0.001$, Pearson-R=0.75) (Supplementary Fig. S5). Fecal *p*-cresol and fecal *p*-cresol sulfate did not show a significant correlation with plasma *p*-cresol sulfate (Supplementary Fig. S6-7), which suggests that gut permeability or other factors may affect how much *p*-cresol leaves the gut. *p*-cresol was not detected in plasma, which is not surprising since it has very low solubility in aqueous solutions. We performed correlation analyses with previously published taxonomic and metabolic pathways (KEGG orthologs) (Nirmalkar et al., 2022), but did not observe statistically significant correlations.

Discussion

A distinct metabolic signature has been consistently observed in plasma and fecal samples collected from children with ASD (Kang et al., 2020; Needham et al., 2021). While several underlying biochemical pathways and environmental factors contribute to these differences, MTT not only improved GI and behavioral symptoms associated with ASD (Kang et al., 2017, 2019) but also lead to a convergence of ASD metabolite profiles towards those observed in TD samples (Kang et al., 2020). Reanalysis of absorbance peaks by Metabolon Inc. using a more

comprehensive database, and improvement in our data analysis pipeline allowed for an increase in the numbers of metabolites detected in plasma and feces (Supplementary Fig. S2) compared to our previous measurements (Kang et al., 2020). Through reanalysis, we identified 844 metabolites in plasma, and 825 metabolites in feces (Fig. 2), which is a substantial increase in identified metabolites from the original study. Furthermore, several biochemical compounds showed notable shifts in plasma and fecal metabolic profiles after MTT in ASD children. Following treatment, 20 metabolites in plasma and 22 in feces that whose intensities were initially dissimilar to TD became similar to TD (Fig. 3).

For plasma metabolites, we found similar results to Kang et al., 2020 for bilirubin (an oxidant), iminodiacetate (xenobiotic and a glycine derivative) (Lin et al. 2019), and sarcosine (a glycine derivative, and beneficial against depression and schizophrenia) (Tsai et al., 2004). The relative intensities of these compounds were significantly lower at baseline but increased significantly after MTT in children with ASD (Fig. 2). Also, the intensity of caprylate (8:0, a medium-chain fatty acid) was higher at baseline and significantly decreased after MTT. The analysis for these metabolites shows the consistency of the data previously reported by Kang et al. (2019) and improvement of plasma metabolites profiles after MTT in children with ASD. However, with this re-analysis we detected some additional important metabolites, such as caproate (6:0, a medium-chain fatty acid), phospholipids such as glycerophosphocholine (GPC), phosphoethanolamine (PEA), glycerophosphoethanolamine (GPEA), and 2-palmitoyl-GPC (16:0). The relative intensity of these metabolites was higher at baseline and significantly decreased after MTT in children with ASD (Fig. 3A). Kang et al. postulated that a decrease in medium-chain fatty acids, such as caprylate to be correlated with an improvement in ASD symptoms (Kang et al., 2020).

Phospholipids are lipids that are highly abundant in neuronal membranes (Joensuu et al., 2020), and they are an important source of energy and provide long-chain polyunsaturated fatty acids (LC-PUFAs). Some research suggests that dysregulated phospholipid metabolism may be a biological component of neurodevelopmental disorders like ASD (Pastural et al., 2009; El-Ansary et al. 2011; Brown et al., 2011; Needham et al., 2021). Increased sphingolipid-1-PO_4, a type of phospholipid that was observed in ASD serum compared to TD (Wang et al., 2016). In our fecal samples, relative intensities sphingosines N-palmitoyl-sphingosine (d18:1/16:0), N-stearoyl-sphingosine (d18:1/18:0), and N-oleoyl-sphingosine (d18:1/18:0) were significantly

higher at ASD baseline compared to TD but after MTT, they significantly decreased, and their abundance became similar to TD (Fig. 3B). These lipids are important for phospholipid metabolism and mitochondrial function (Tamji & Crawford, 2011). However, the differential abundance of these mitochondrial markers in fecal and plasma metabolites in children with ASD suggests potential mitochondrial dysfunction in children with ASD and may contribute to ASD severity (Needham et al., 2021; Pastural et al, 2009; Rossignol & Frye, 2012; Griffiths & Levy, 2017; Lombard, 1998).

In plasma, GPC (phospholipid) levels were significantly higher in children with ASD compared to their TD counterparts. While GPC levels decreased following MTT, the change was not statistically significant (Fig 3A). Similarly, Needham et al reported that GPC was significantly higher in ASD children's plasma samples and was associated with mitochondrial dysfunction (Needham et al., 2021). GPC is a phospholipid-containing choline found in human milk (Dangat & Johsi, 2023) and is an important nutrient for memory, mood, and muscle regulation). However, administration of GPC has been shown to improve cognition against dementia and Alzheimer's disease (AD) (Amenta et al., 2014; Sagaro et al., 2023). In animal studies, administration of GPC was found to release acetylcholine in rat brains, elevate dopamine and serotonin levels in the frontal cortex, and enhance cognitive function (Traini et al., 2020). Despite the beneficial roles of phospholipids containing choline's, increased GPC levels in our samples from children with ASD suggest dysregulation in phospholipids metabolism, and it has been reported that elevated levels of GPC+phosphocholines (PC) can be associated with bipolar disorder patients (Cao et al., 2017).

In feces, *p*-cresol and *p*-cresol sulfate have consistently emerged as metabolites of interest due to their elevated presence in the urine and feces of children diagnosed with ASD (Altieri et al., 2011, Chen et al., 2013; Kang et al., 2018; Daneberga et al., 2022; Diémé et al., 2015; Emond et al., 2013; Gevi et al 2016; Gevi et al., 2020, Li et al., 2018; Mussap et al., 2020; Osredkar et al., 2023; Perisco & Napolioni 2013, Piras et al., 2022; Tevzadze et al., 2017; Timperio et al., 2022). The primary source of *p*-cresol in the body is its production by gut bacteria as an antimicrobial compound (Saito et al., 2019). *p*-cresol is produced *via* tyrosine metabolism and is considered a uremic toxin (Candleliere et al., 2022). To eliminate *p*-cresol from the body, the host liver carries out sulfation, forming *p*-cresol sulfate, increasing its

solubility so that it can be more easily excreted from the body (Gryp et al., 2017; Spanogiannopoulos et al., 2016; Clayton et al., 2009).

We observed that intensity of fecal *p*-cresol sulfate was significantly higher at ASD baseline compared to TD (Fig. 3B, 7A). After MTT, *p*-cresol sulfate decreased significantly, and its abundance became more similar to TD (Fig. 3B, Fig. 7A). However, we did not observe significant differences or changes between groups for *p*-cresol in feces, *p*-cresol sulfate for plasma, and *p*-cresol was not detected in plasma. Recent mouse studies have shown that when *p*-cresol was administered, mice exhibited social behavior deficits and more repetitive behavior relative to controls (Bermudez-Martin et al., 2021), and subsequent fecal microbiota transplant from healthy mice led to normalization of symptoms (Bermudez-Martin et al., 2021). These findings suggest that *p*-cresol and/or *p*-cresol sulfate may contribute to the pathophysiology of autism. Current results led to a hypothesis that MTT helps to reduce the *p*-cresol sulfate levels by remodeling the gut microbial ecology in ASD children and allowing the growth of microbiota from healthy donors.

Another sulfated metabolite that has been associated with ASD in other studies, is 4-ethylphenyl sulfate (4EPS) (Hsiao et al., 2013; Needham et al., 2021; Stewart Campbell et al., 2022). While we did not detect 4EPS in fecal samples and also did not observe significant differences or changes between groups in plasma samples. Recent clinical trials in children with ASD + AB-2004, an adsorbent, showed decreased *p*-cresol sulfate and 4EPS levels in urine. These changes occurred alongside improvements in ASD-like behaviors, suggesting 4EPS and *p*-cresol sulfate could be correlated with behavioral severity in ASD (Stewart Campbell et al., 2022).

Our most robust multivariate FDA model was composed of five metabolites from either plasma or fecal samples; *p*-cresol sulfate (feces), hydroxyproline (feces), caprylate (plasma), indole-propionate (plasma), and bilirubin (Z, Z) (plasma), that were different at baseline vs TD and became similar after MTT (Fig. 3A-B). Fecal *p*-cresol sulfate emerged as a recurring component in many of the top-performing multivariate FDA models. Univariate analysis demonstrated strong differences in the composition of *p*-cresol sulfate between the ASD baseline and TD groups, achieving an AUROC of 0.76. These differences were utilized in the linear

multivariate models relying on this variable, highlighting *p*-cresol sulfate as a potentially relevant biomarker for ASD in feces. Lower caprylate (plasma), and higher indole-propionate (plasma), and higher bilirubin (Z, Z) (plasma) were also observed in the previous study (Kang et al., 2020) at baseline and after MTT, relative intensity became similar to TD levels. Indole-propionate, a gut metabolite has been found to be associated with ASD in humans (Yu et al., 2020) and animal models (Wang et al., 2023; Jiang et al., 2024). Hydroxyproline, another FDA model-identified metabolite (Fig. 4D), was previously identified at significantly higher relative intensity in the plasma of children with ASD (Chen et al., 2024).

Combining plasma and fecal metabolite datasets resulted in higher cross-validated accuracy than using either dataset alone, potentially as a consequence of complimenting biological insight provided by each source. The FDA models developed for the reanalysis demonstrated an accuracy of 95%, outperforming previous work with fecal metabolites from the same study, which achieved an accuracy of 94.7% (Qureshi et al., 2020). This prior work also highlighted that after MTT treatment, the predictive accuracy and average AUROC of fecal metabolites decreased substantially. This pattern was observed in the metabolomic panels composed of both plasma and fecal samples in this study as well, suggesting that improvements in several metabolites may have compromised their predictive validity. Future studies should aim to confirm the impact of MTT on ASD using a larger cohort with a placebo arm to ensure robust validation and broader clinical relevance.

In conclusion, the reanalysis of plasma and fecal metabolites revealed additional 223 plasma and 159 fecal metabolites compared to the previous report by Kang et al., 2020. The contrast in metabolite composition before and after MTT groups provides insight on metabolites with potential biological relevance in children with autism and/or GI issues. This research underscores the value of re-analysis when databases improve and the importance of investigating non-behavioral features in ASD diagnosis and provides valuable insights into the intricate metabolic landscape of this complex disorder.

Using different analysis methods, we consistently observed that sarcosine, iminodiacetate, caproate, and caprylate in plasma were significantly different in ASD vs TD, as reported previously. However, we also identified phospholipids such as glycerophosphoethanolamine (GPE), glycerophosphocholine (GPC), and phosphoethanolamine

in plasma, as well as fecal sphingosines N-palmitoyl-sphingosine (d18:1/16:0), N-stearoyl-sphingosine (d18:1/18:0), and N-oleoyl-sphingosine (d18:1/18:0), which were significantly different at baseline compared to typically developing (TD) individuals and/or changed after MTT. Moreover, we observed that fecal *p*-cresol sulfate was higher at baseline compared to TD and decreased significantly after MTT.

Notably, *p*-cresol sulfate in feces was consistently found across our different statistical methods as a potential biomarker before and after MTT in children with ASD. This suggests the potential of microbially-produced metabolite in affecting ASD symptoms. Larger cohort and placebo-controlled trials are necessary to validate our findings and unravel the mechanisms underlying MTT in ameliorating aberrant metabolomic profiles associated with ASD. Furthermore, insights into the underlying biochemical pathways will facilitate the development of more tailored treatment strategies.

Additional Information: The Supplementary Material for this article can be found online at:

Authors Contributions

Conceptualization, K.N., D.W.K., J.B.A., R.K.B.; Data curation, K.N, F.Q., J.B.A., R.K.B.; Formal analysis, K.N, F.Q., J.B.A., R.K.B.; Funding acquisition, D.W.K., J.B.A., R.K.B.; Investigation, K.N, F.Q., J.B.A., R.K.B.; Methodology, K.N., J.H., J.B.A., R.K.B.; Project administration, R.K.B., J.B.A.; Software, K.N.; F.Q.; Supervision, D.W.K., J.H., J.B.A., R.K.B.; Visualization, K.N., F.Q.; Writing—original draft, K.N., F.Q., J.B.A., R.K.B.; Writing—review & editing, , K.N., F.Q., D.W.K., J.H., J.B.A., R.K.B.

Disclosure/Conflict of interest

K.N., J.B.A., D.-W.K., and R.K.-B. have pending/approved patents related to the usage of fecal microbiota transplant and/or probiotics for various conditions, including autism, and have filed an invention disclosure on the results presented here. J.B.A., R.K.-B., and D.-W.K. received funding from Crestovo/Finch Therapeutics for FMT research. J.B.A. and R.K.-B. were part-time consultants for Crestovo. M.S. has shares in Finch Therapeutics. The other authors do not have any financial conflicts of interest.

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Figures:

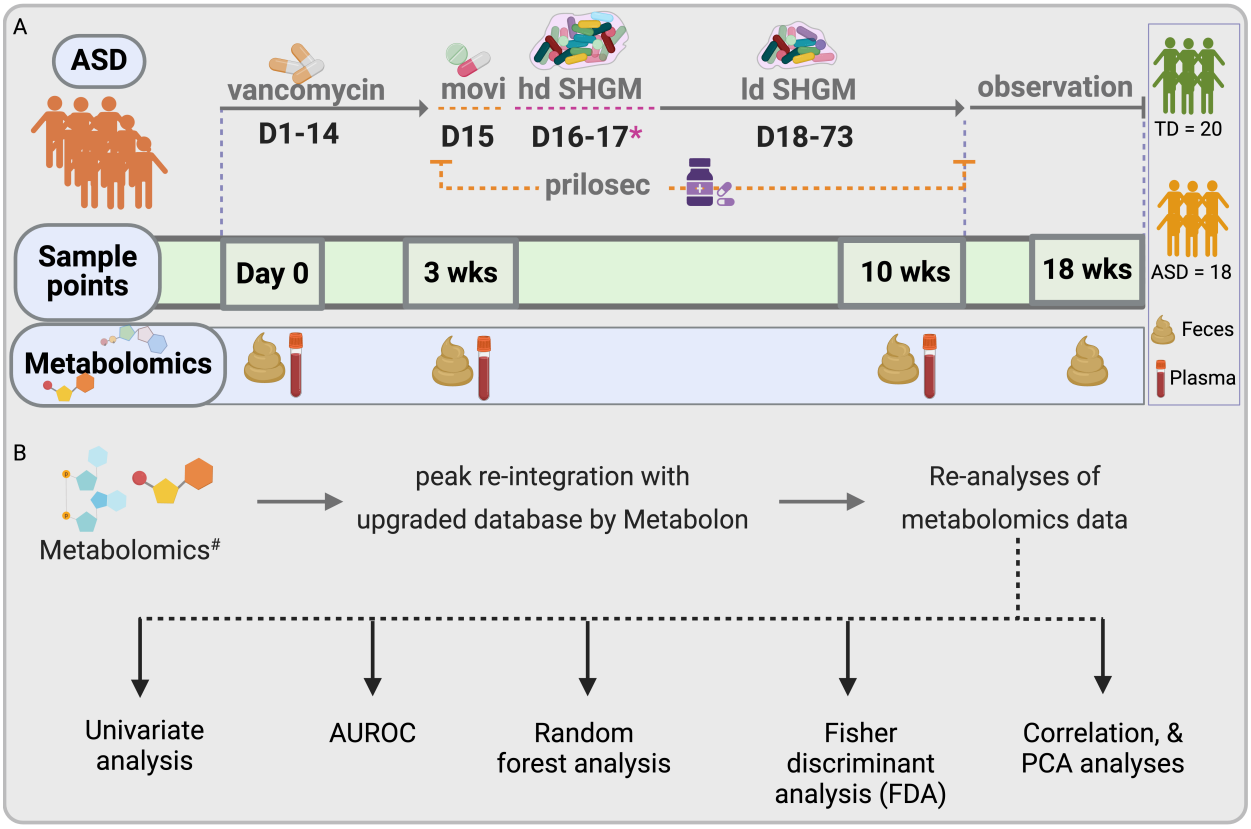

Figure 1: The study design of microbiota transplant therapy for fecal and plasma metabolomics (A) and the overview of the data analyses for fecal and plasma metabolites (B).

Plasma was collected right before high-dose microbiota and feces were collected right after high-dose microbiota.

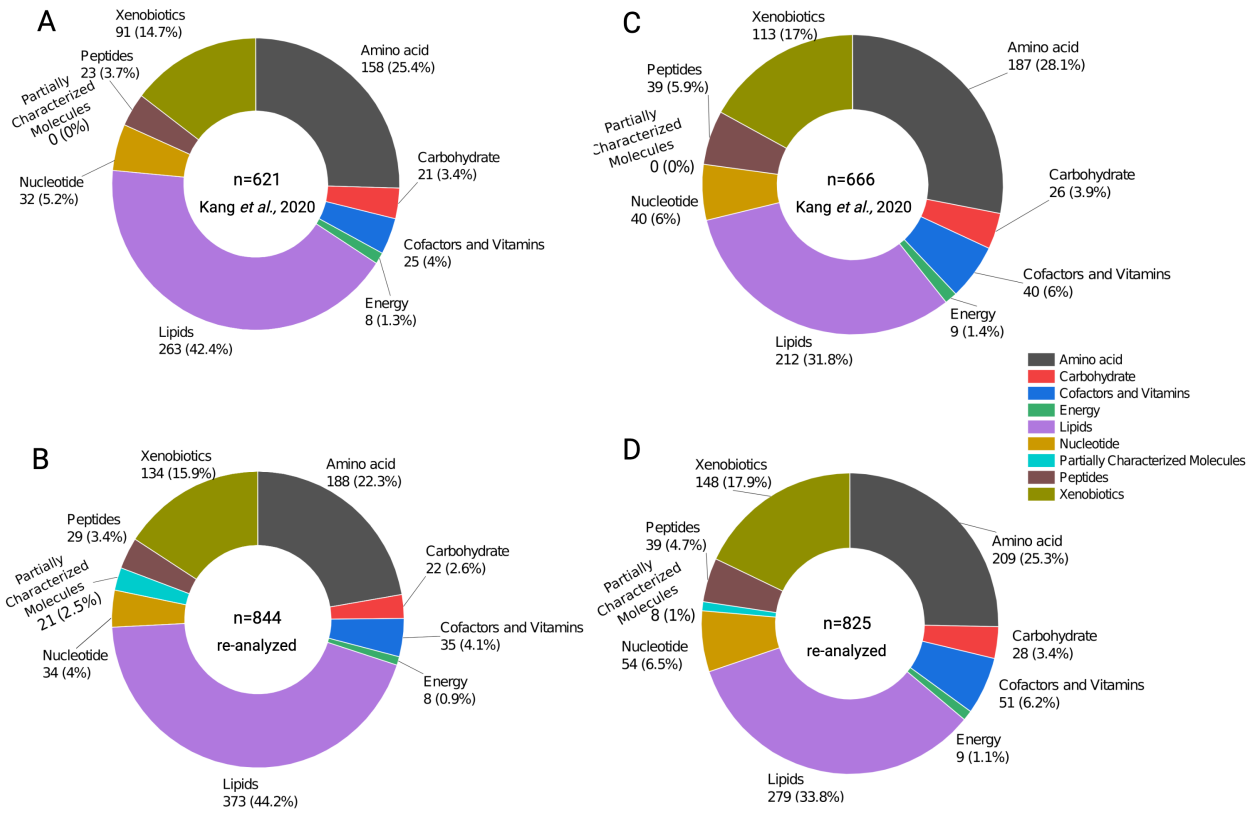

Figure 2. An overview of plasma metabolomics with the previous A) and re-analyzed B), fecal metabolomics with the previous C) and re-analyzed D) of peaks. The pie charts show the total number of metabolites identified and the type of metabolites in plasma and fecal samples.

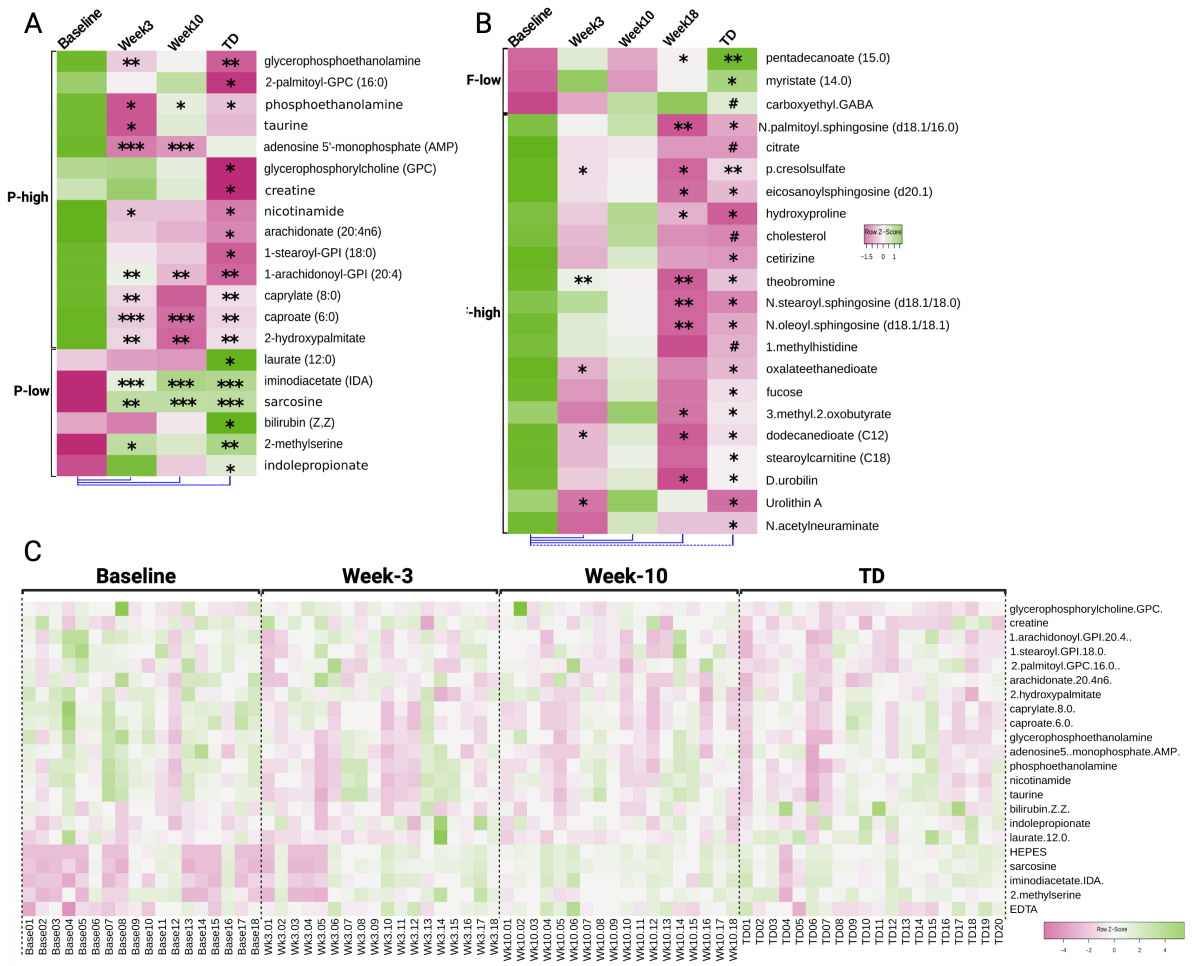

Figure 3. Univariate comparison of the (z-score) relative intensity of plasma (A) and fecal (B) metabolites in children with ASD and comparisons after MTT and with TD. For the plasma metabolites (A), a longitudinal paired-wise univariate analysis was performed at baseline vs. week3, week10 and cross-sectional unpaired comparison vs. TD. Similarly, for the fecal metabolites (B), a longitudinal paired-wise univariate analysis was performed (shown in solid blue lines at the bottom of the heatmap) at baseline vs. week3, week10, week18 and cross-sectional unpaired comparison vs. TD (shown in dashed blue line). (C) heatmap (z-score) for each participant with 20 plasma metabolites that were significantly changed or were different in children with ASD at baseline compared with MTT and with TD groups in Figure 3A. Week18 time was not available for the plasma. See Supplementary Fig. S1 for fecal metabolites heatmap for each participant. The median of relative intensities in plasma (A) and feces (B) was used to construct the heatmap for each group. *Single asterisk indicates $p < 0.05$, **double asterisks indicate $p < 0.01$, triple *** asterisks indicate $p < 0.001$. # indicates the statistically significant ($p<0.05$) metabolites after log-transformation. All p-values are FDR corrected. ASD: Autism Spectrum Disorders, TD: Typically Developing.

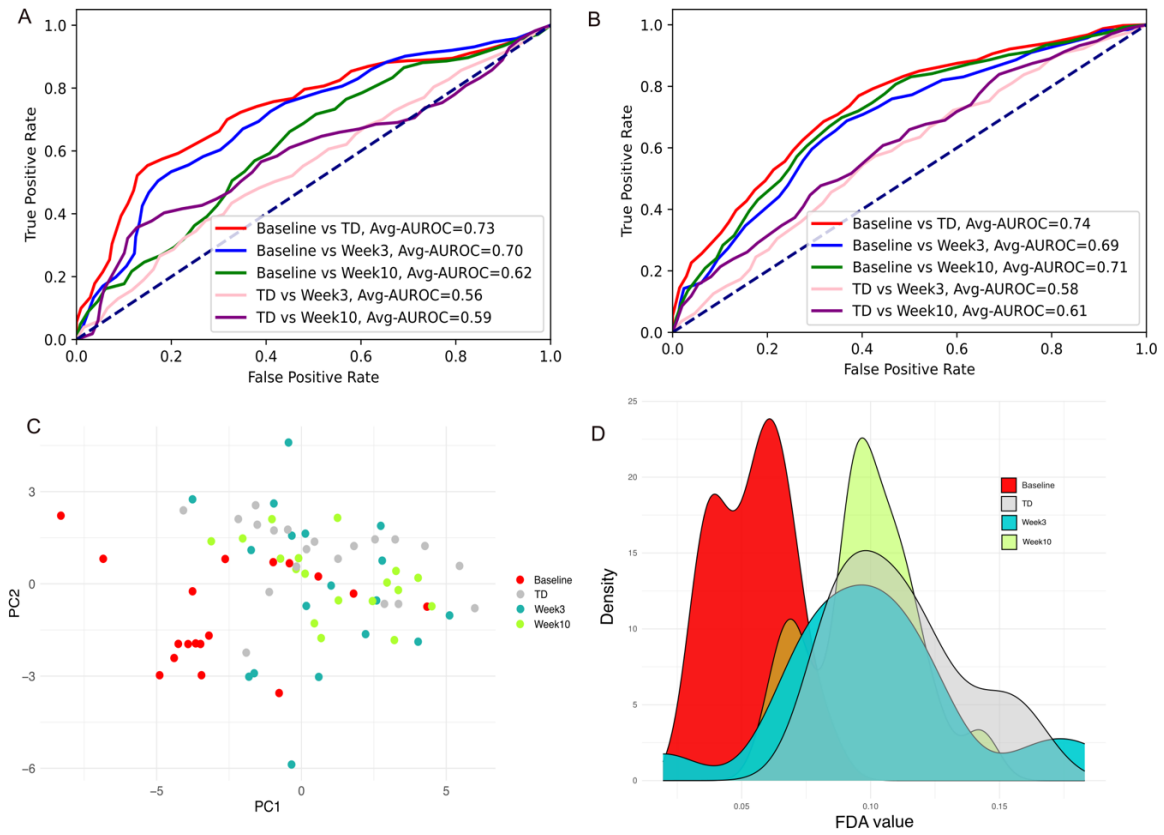

Figure 4. An average Receiver operating characteristic curves (AUROC) of A) plasma, B) fecal metabolites, C) principal component analysis (PCA) of plasma metabolites, and D) FDA analysis of fecal and plasma metabolites (*p*-cresol sulfate, caprylate (C:8) bilirubin (Z,Z), indolepropionate and hydroxyproline) using probability density curves associated with FDA scores before and after MTT in ASD, and TD children. For FDA analysis, true positive vs false positive rate was determined across all threshold values to characterize between ASD Baseline against all possible alternative groups. For AUROC and PCA plots, 22 fecal and 20 plasma metabolites (presented in Figure 3) were used. Refer to Supplementary Figure S2 for PCA plot for fecal metabolites. ASD – autism spectrum disorder, MTT - microbiota transfer therapy, TD - typically developing, FDA - Fisher discriminant analysis.

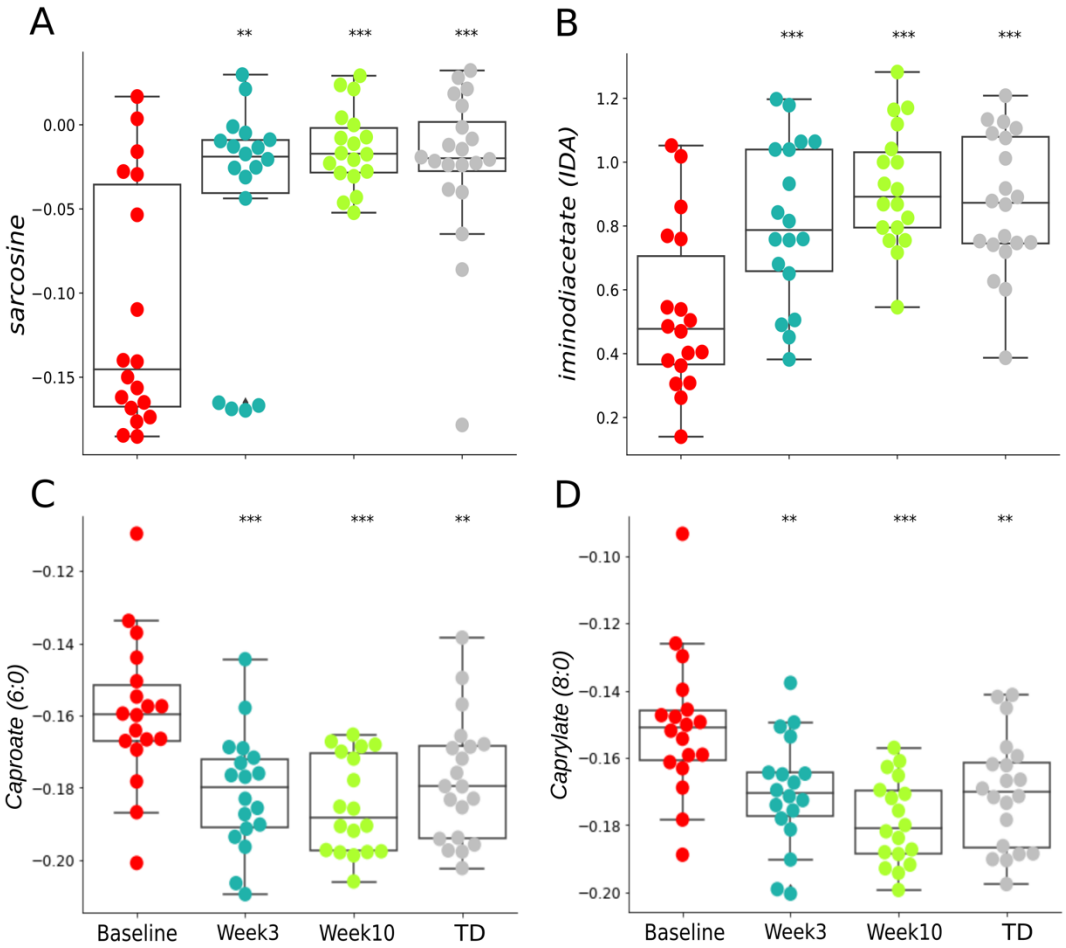

Figure 5. Univariate comparison of the relative intensity (after log10 transformation) in plasma for A) Sarcosine, B) Iminodiacetate (IDA), C) Caproate (6:0), and D) Caprylate (8:0) at ASD baseline vs. MTT (week3, week10) and TD. Each colored dot represents one ASD individual, and grey colored dots represent TD. Asterisks represent significant differences between ASD baseline, and the other groups (*Single asterisk indicates $p < 0.05$, **double asterisks indicate $p < 0.01$, ns not significant, all p-values are FDR corrected). ASD: Autism Spectrum Disorders, TD: Typically Developing.

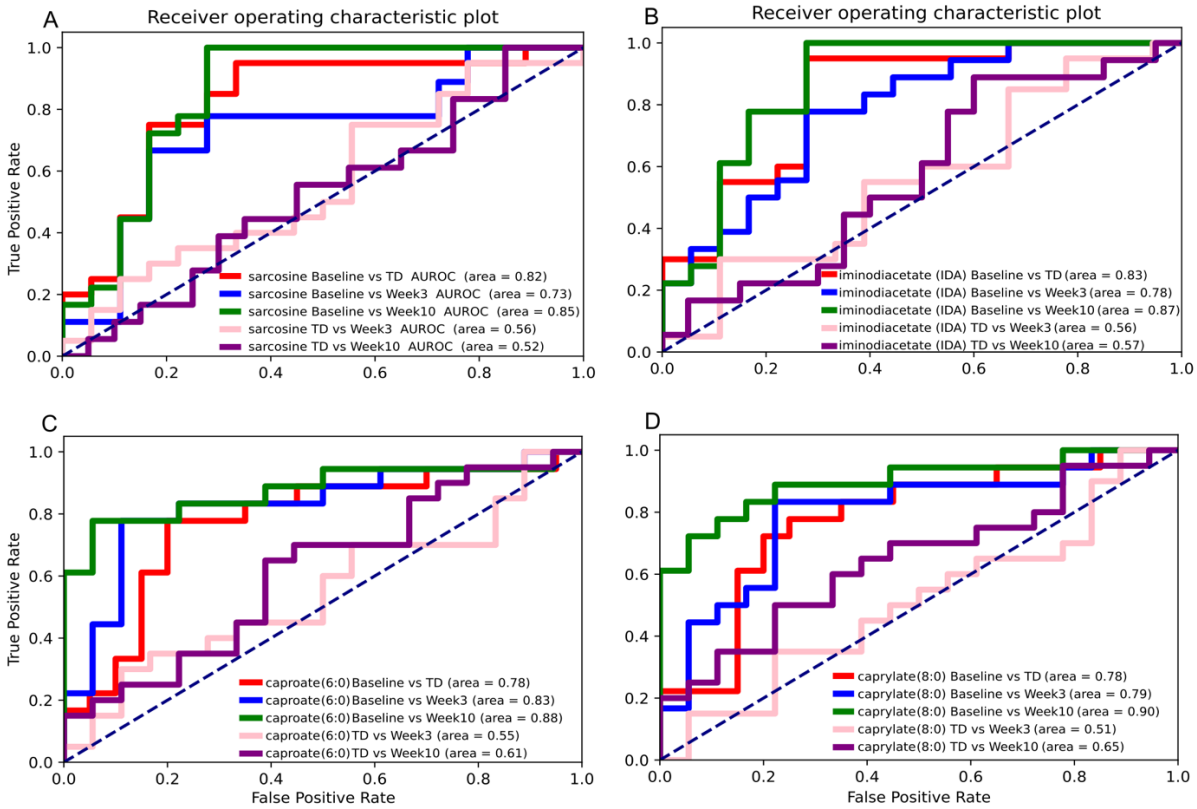

Figure 6. Receiver operating characteristic curves (AUROC) of A) Sarcosine, B) Iminodiacetate (IDA), C) Caproate (6:0), and D) Caprylate (8:0) for ASD baseline vs. MTT (week3, week10) and TD. True positive vs false positive rate was determined across all threshold values to characterize between ASD Baseline against all possible alternative groups.

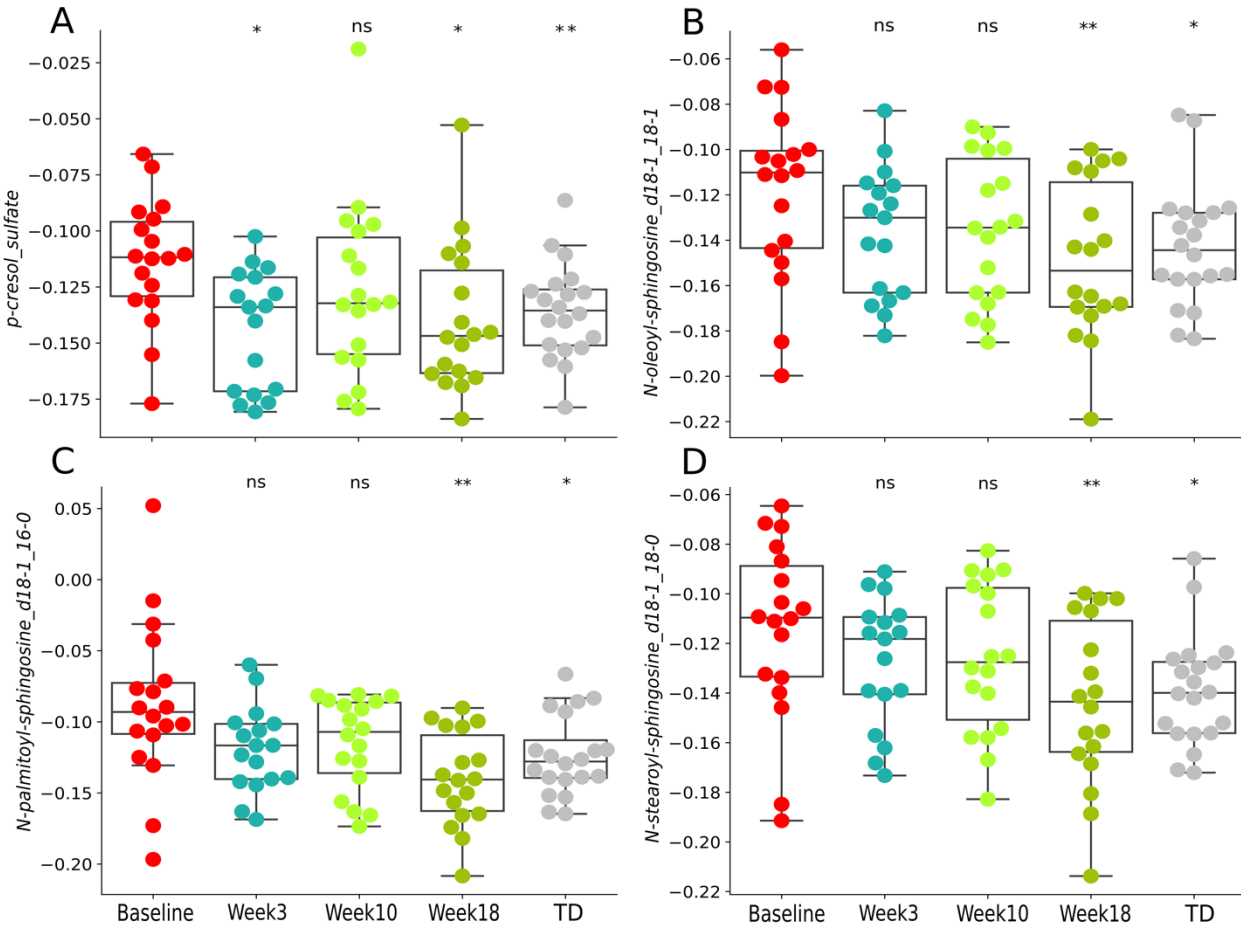

Figure 7. Univariate comparison of the relative intensity (after log10 transformation) in feces for A) p-cresol sulfate, B) N-oleoyl-sphingosine (d18:1/18:1), C) N-palmitoyl-sphingosine (d18:1/16:0), and D) N-stearoyl-sphingosine (d18:1/18:0) at ASD baseline vs. MTT (week3, week10, week18) and TD. Each colored dot represents one ASD individual and grey colored dots represent TD. Asterisks represent significant differences between ASD baseline and the other groups (*Single asterisk indicates $p < 0.05$, **double asterisks indicate $p<0.01$, ns not significant, all p-values are FDR corrected). ASD: Autism Spectrum Disorders, TD: Typically Developing.

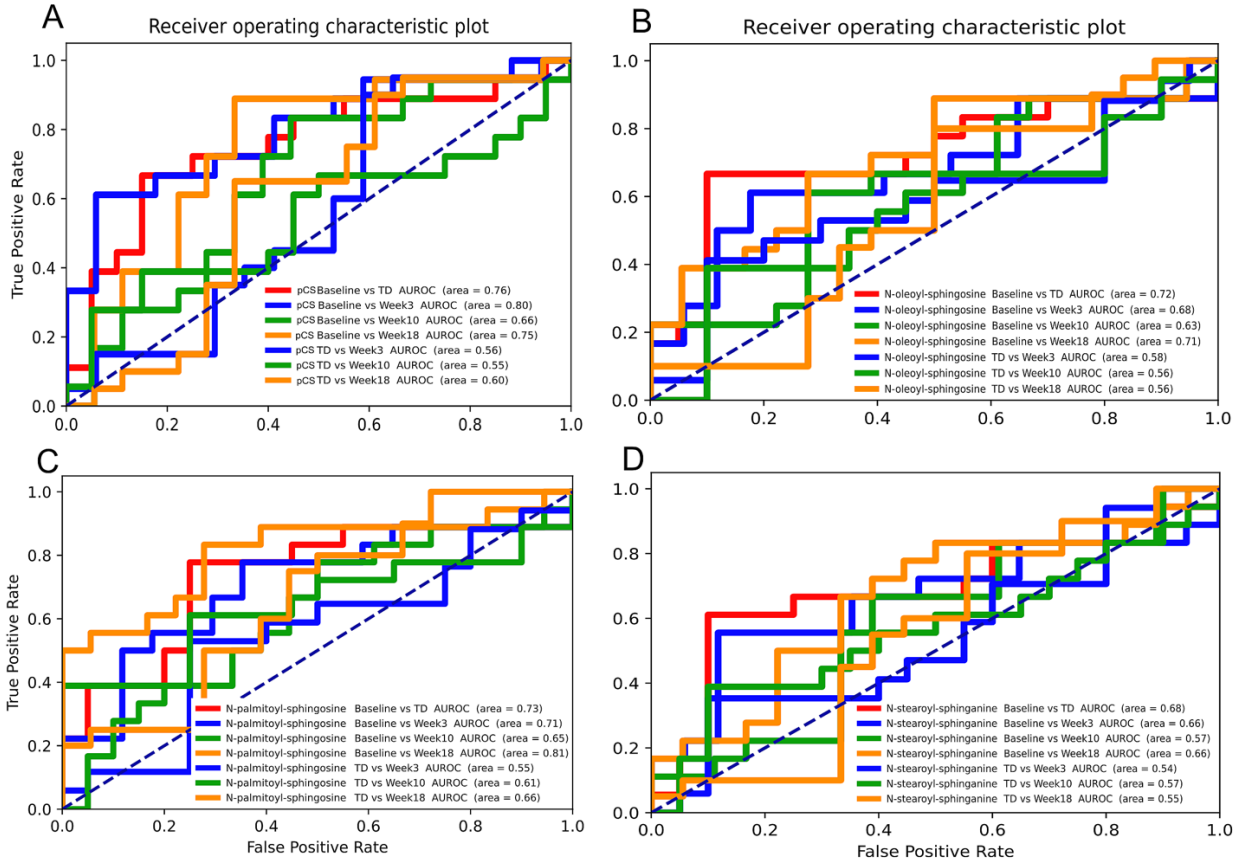

Figure 8. Receiver operating characteristic curves (AUROC) of A) p-cresol sulfate, B) (N-oleoyl-sphingosine d18:1/18:1), C) N-palmitoyl-sphingosine (d18:1/16:0), and D) N-stearoyl-sphingosine (d18:1/18:0) at ASD baseline vs. MTT (week3, week10, week18) and TD. True positive vs false positive rate was determined across all threshold values to characterize between ASD baseline against all possible alternative groups.

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