RESEARCH ARTICLE

A Metabolomics Approach to Screening for Autism Risk in the Children's Autism Metabolome Project

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Autism spectrum disorder (ASD) is biologically and behaviorally heterogeneous. Delayed diagnosis of ASD is common and problematic. The complexity of ASD and the low sensitivity of available screening tools are key factors in delayed diagnosis. Identification of biomarkers that reduce complexity through stratification into reliable subpopulations can assist in earlier diagnosis, provide insight into the biology of ASD, and potentially suggest targeted interventions. Quantitative metabolomic analysis was performed on plasma samples from 708 fasting children, aged 18 to 48 months, enrolled in the Children's Autism Metabolome Project (CAMP). The primary goal was to identify alterations in metabolism helpful in stratifying ASD subjects into subpopulations with shared metabolic phenotypes (i.e., metabotypes). Metabotypes associated with ASD were identified in a discovery set of 357 subjects. The reproducibility of the metabotypes was validated in an independent replication set of 351 CAMP subjects. Thirty-four candidate metabotypes that differentiated subsets of ASD from typically developing participants were identified with sensitivity of at least 5% and specificity greater than 95%. The 34 metabotypes formed six metabolic clusters based on ratios of either lactate or pyruvate, succinate, glycine, ornithine, 4-hydroxyproline, or α-ketoglutarate with other metabolites. Optimization of a subset of new and previously defined metabotypes into a screening battery resulted in 53% sensitivity (95% confidence interval [CI], 48%–57%) and 91% specificity (95% CI, 86%–94%). Thus, our metabolomic screening tool detects more than 50% of the autistic participants in the CAMP study. Further development of this metabolomic screening approach may facilitate earlier referral and diagnosis of ASD and, ultimately, more targeted treatments. ***Autism Res*** *2020, 00: 1–16.* © 2020 The Authors. *Autism Research published by International Society for Autism Research* published by Wiley Periodicals LLC.

Lay Summary: Analysis of a selected set of metabolites in blood samples from children with autism and typically developing children identified reproducible differences in the metabolism of about half of the children with autism. Testing for these differences in blood samples can be used to help screen children as young as 18 months for risk of autism that, in turn, can facilitate earlier diagnoses. In addition, differences may lead to biological insights that produce more precise treatment options. We are exploring other blood-based molecules to determine if still a higher percentage of children with autism can be detected using this strategy.

Keywords: autism spectrum disorder; amino acids; energy metabolism; metabolomics; biomarkers; mitochondria; risk

Introduction

Autism spectrum disorder (ASD) is a clinically and etiologically heterogeneous neurodevelopmental condition [Jeste & Geschwind, 2014; Lord, Elsabbagh, Baird, & Veenstra-Vanderweele, 2018]. The average age of ASD diagnosis in the United States is over 4 years [Baio et al., 2018; Hall-Lande, Esler, Hewitt, & Gunty, 2018] and is based on behaviorally assessed alterations in social interaction and persistent repetitive behaviors or circumscribed interests [American Psychiatric Association, 2013]. There is substantial evidence that earlier diagnosis of ASD improves outcomes by expediting behavioral therapy that leads to higher cognitive and social function [Dawson et al., 2010; Estes et al., 2015]. This has the added benefit of reducing the financial and emotional burden on families and society [Lavelle et al., 2014].

As a result of the high global population prevalence (1%–2%) of ASD [Elsabbagh et al., 2012; Lyall et al., 2017], its substantial impact on affected individuals

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and their families [Buescher, Cidav, Knapp, & Mandell, 2014; Muskens, Velders, & Staal, 2017], and the potential benefit of early intervention, screening for ASD is recommend for children at 18 and 24 months during routine pediatric visits. Additional assessment is carried out if a child is deemed to be at high risk for ASD [Hyman et al., 2020; Monteiro, Dempsey, Berry, Voigt, & Goin-Kochel, 2019]. The American Academy of Pediatrics recommends that children who fail a screening test should be referred to specialists who are trained to make a diagnosis of ASD [Hyman et al., 2020].

While parental questioning is widely used as a screen for ASD, a number of studies have indicated that this strategy is not optimal [Monteiro et al., 2019; Zwaigenbaum & Maguire, 2019]. The Modified Checklist for Autism in Toddlers with Follow-Up (M-CHAT/F), for example, is reported to have a sensitivity of only 38.8% and a positive predictive value of 14.6% [Guthrie et al., 2019]. Thus, this widely used screening tool detects less than 40% of children who will go on to attain a diagnosis of ASD, and less than 15% of the children who are positive on the test actually end up with a diagnosis of ASD. Failure to identify a child with risk for ASD during screening will lead to delayed diagnosis [Monteiro et al., 2019].

There has been intense interest in discovering easily implementable biomarkers that support screening, diagnosis, and targeted intervention of ASD [McPartland, 2017; Riedl, Gieger, Hauner, Daniel, & Linseisen, 2017]. Diverse modalities of biomarkers have been investigated including neuroimaging, EEG, eye tracking, pupillary reflex, and transcriptomic, proteomic, and metabolomics markers [Bridgemohan et al., 2019; Broek et al., 2014; Ruggeri, Sarkans, Schumann, & Persico, 2014; Shen et al., 2019]. Potential metabolic biomarkers of ASD have been identified, mainly in blood or urine, using a variety of analytical approaches that have suggested that a range of metabolic processes are altered in ASD [De Angelis et al., 2013; Glinton & Elsea, 2019; Lanz et al., 2013; Ming, Stein, Barnes, Rhodes, & Guo, 2012; Ming et al., 2005; Orozco, Hertz-Picciotto, Abbeduto, & Slupsky, 2019; Yap et al., 2010].

Metabotyping is subtyping based on shared metabolic phenotypes identified using metabolic biomarkers. Metabotyping using metabolic biomarkers associated with ASD can enable stratification of the disorder into distinct subpopulations based on a common metabolic dysregulation identified by the biomarker. Stratification of ASD using metabotype-based tests can lead to underlying biological differences among those with ASD and, in turn, potentially to targeted therapeutic intervention for individuals with a specific metabotype [Wolfers et al., 2019; Yap et al., 2010].

We conducted the Children's Autism Metabolome Project (CAMP) to identify metabolic dysregulations associated with ASD. CAMP, the largest metabolomics study of children with ASD to date, was designed to reproducibly identify metabotypes associated with ASD. We recruited 1,102 children between the ages of 18 months and 4 years from eight clinical sites spread across the United States. Of these, 708 had a diagnosis of ASD or were typically developing (TYP) and were able to contribute blood samples that met quality control standards for metabolic analyses. Previous analysis of CAMP metabolomics data identified a group of plasma metabolites in autistic children that were negatively correlated with plasma branched chain amino acids (BCAAs). Imbalances in the concentrations of the amino acids glycine, glutamine, and ornithine relative to the BCAAs identified ASD-associated amino acid metabotypes (AADMs) that were present in 17% of the ASD subjects [Smith et al., 2019].

In the current study, we quantitatively assessed 39 metabolites associated with amino acid and energy metabolism in an attempt to expand the identification of metabolic subpopulations of children with ASD. This set of metabolites was chosen based on our pilot studies [Smith et al., 2019; West et al., 2014] and published research related to abnormalities of biochemical processes noted in ASD related to purine metabolism and mitochondrial bioenergetics [Broek et al., 2014; De Rubeis et al., 2014; László, Horváth, Eck, & Fekete, 1994; Shen et al., 2019; Yehia et al., 2019]. The current work presents the results of this metabolomic analysis and explores the potential of these metabotype tests as another step toward creating a metabolomic screening platform to determine risk for ASD in young children.

Methods

CAMP Participants

The case–control CAMP study consented 1,102 children, ages 18 to 48 months, from eight centers across the United States from August, 2015 to January, 2018 (ClinicalTrials.gov Identifier: NCT02548442). The eight centers included Children's Hospital of Philadelphia, Cincinnati Children's Hospital, The Lurie Center at Massachusetts General Hospital, The Melmed Center, The MIND Institute, University of California—Davis, Nationwide Children's Hospital, The University of Arkansas for Medical Sciences, and Vanderbilt University Medical Center. Children were excluded from the study if they were previously diagnosed with a genetic condition. Children who had recognized serious neurological, metabolic, psychiatric, cardiovascular, or endocrine system disorders were also excluded. Children exhibiting signs of illness within 2 weeks of enrollment were rescheduled for blood collection. All participants underwent medical and behavioral examinations. Metadata were obtained about the child's gestational history, birth, developmental,

medical and immunization histories, dietary supplements, and medications. Brief parental medical histories were also obtained. The Autism Diagnostic Observation Schedule-Second Version (ADOS-2) assessment was performed by research reliable clinicians to confirm ASD diagnoses. A developmental quotient (DQ) was derived from The Mullen Scales of Early Learning which was administered to all children. A child was diagnosed as ASD if the ADOS-2 comparison severity score was 4 or higher. A child was designated as typical if the DQ was greater than 70 and was not diagnosed by a clinician as having a developmental disorder. Specimens of plasma were collected and processed as previously described [Smith et al., 2019]. The study protocol was approved and monitored by institutional review boards at each of the clinical centers. Written informed consent from a parent or legal guardian was obtained, and a small monetary stipend was provided for each participant. Of the 1,102 consented children, 645 had a diagnosis of ASD and 255 were TYP. Of the 900 subjects receiving these diagnoses, 708 provided plasma samples meeting study and quality control criteria for inclusion in this analysis (Table 1).

Assignment of Subjects to Discovery and Replication Sets

The discovery set was established to measure metabotype-positive populations with a sensitivity of 8% with a lower confidence limit of 3% and specificity of 95% with a lower confidence limit of 85% under an alpha of 5% and a power of at least 0.90 [Flahault & Thomas, 2005]. The replication set of subjects was established and analyzed once enough subjects were recruited to match the demographic composition of the discovery set (Table 1). Randomization of available CAMP participants was performed within study sets to maintain a prevalence of ASD of approximately 70%. Randomization was restricted by age, DQ, and sex to maintain discovery set demographic values in the replication set.

Phlebotomy Procedures

Blood was collected by venipuncture into 6 ml sodium heparin tubes placed on wet ice from subjects who had not eaten for at least 12 hr. Plasma was obtained by centrifugation (1200*g* for 10 min) and frozen to −70°C within 1 hr. Hemolysis of blood samples was measured spectrophotometrically in plasma [Noe, Weedn, & Bell, 1984]. Plasma samples with hemoglobin levels >600 mg/dl were excluded from analyses. Values for the analytes xanthine, uric acid, or hypoxanthine (which are more sensitive to hemoglobin interference) were omitted when hemoglobin levels exceeded 200 mg/dl.

Quantitative Analysis of Candidate Metabolites Using Liquid Chromatography–Tandem Mass Spectrometry

Three quantitative liquid chromatography–tandem mass spectrometry (LC–MS/MS) methods measuring a total of 39 unique endogenous metabolites and 37 stable isotope-labeled internal standards (Table S1) were analytically validated in compliance with FDA and CLSI guidance for bioanalytical method validation (Lynch, 2016; U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research [CDER], 2018). Following analytical validation, the quantitative assays were used to measure biological amines [Smith et al., 2019], purines, and carboxylic acid-containing analytes in participant samples. Detailed information about the sample preparation, detection, and quantification of metabolites are described in the Appendix S1. Analyte quantification was performed using an Agilent Technologies G6490 Triple Quadrupole Mass Spectrometer and a Waters Xevo TQ-S micro, IVD mass

Table 1. CAMP Study Population Demographics by Study Set

Demographic	CAMP study sets		
Study set	Discovery	Replication	Total
No. of ASD children	253	246	499
No. of TYP children	104	105	209
Total	357	351	708
ASD vs TYP prevalence, %	70.9	70.1	70.5
ASD male[a], %	77.9	80.1	79
TYP male[a], %	60.6	58.1	59.3
ASD Age[b], mean (SD), months	35.7 (7.6)	34.5 (8)	35.1 (7.8)
TYP Age[b], mean (SD), months	33.2 (8.5)	31.9 (9)	32.6 (8.7)
Age range, months	18–48	18–48	18–48
ASD DQ[c], mean (SD)	61.7 (16.9)	63.6 (18)	62.6 (17.5)
TYP DQ[c], mean (SD)	100.1 (15.1)	103.3 (17.4)	101.7 (16.3)
ASD ADOS-2 comparison score, mean (SD)	7.1 (1.9)	7.2 (1.7)	7.1 (1.8)

Note. Superscript letters a,b, and c indicate a comparison with statistically significant difference between ASD and TYP populations (*P*-value <0.05).

spectrometer with appropriate internal standards, calibration ranges, and quality control samples (Appendix S1).

Bioinformatic Analyses

The values of each metabolite or ratio of metabolites were log base 2 transformed and Z-score normalized prior to analyses. Imputation was not performed and missing data were omitted from analysis, reducing the number of samples analyzed for a test statistic. Analysis of covariance, analysis of variance (ANOVA), Welch *T*-tests, and pairwise Pearson correlation analyses were performed on each metabolite or ratio of metabolites. Effect sizes were reported using Cohen's *d* for *T*-tests or generalized eta squared for ANOVA. Dissimilarity measurements (1 – the absolute value of the pairwise Pearson correlation coefficient (ρ) of metabolite ratios) were used to calculate distances for clustering. Hierarchical clustering was performed using the unweighted pair group method with arithmetic mean (UPGMA). Bootstrap analysis of the clustering result was performed using the pvclust package [Suzuki & Shimodaira, 2006]. Clusters were considered significant, and therefore stably identified within repeated sampling, when the unbiased *P*-value was ≥0.95. The independence of subject metadata relative to the metabotypes was tested using the Fisher Exact test statistic, and effect sizes were estimated with Crammer's V. Post hoc evaluation of the responses within metadata variables was performed using an exact binomial test. False discovery rate corrections of *P*-values were performed to control for multiple testing [Benjamini & Hochberg, 1995]. Analyses were conducted using R version 3.6.1 [R Core Team, 2019].

Metabotyping Analysis

A metabotype is a subpopulation of individuals with a shared metabolic characteristic or phenotype that can be distinguished from the larger population [Riedl et al., 2017]. We carried out this study in an attempt to identify metabolic features (i.e., an individual metabolite or ratio of metabolites) that are able to distinguish subpopulations (or metabotypes) of ASD subjects. Potential metabotypes associated with ASD were identified by using a heuristic algorithm that tested whether a metabolite or ratio of metabolites identified a subpopulation of primarily ASD subjects above (or below) a particular quantity of the metabolite or above the threshold in a ratio of metabolites [Smith et al., 2019]. These thresholds were then used to create metabotype tests that identified subjects exceeding the threshold as metabotype-positive and subjects that did not as metabotype-negative. The metabotype tests were established in the discovery set. Diagnostic performance and reproducibility of the metabotype tests were evaluated in the replication set.

Diagnostic performance metrics of sensitivity (detection of ASD) and specificity (detection of TYP) were calculated based on the percentage of ASD or TYP subjects who were positive or negative for a metabotype test. The criteria utilized to accept a metabotype test as being associated with ASD was based on both diagnostic performance and a permutation test to determine if the diagnostic performance values were due to chance. The minimum diagnostic criteria required for a metabotype in the discovery set to be further evaluated in the replication set were at least 5% sensitivity (indicating that at least 5% of the ASD participants were metabotype-positive), at least 95% specificity (indicating that 95% of the TYP participants were metabotype-negative), and the metabotype-positive population was at least 90% ASD. A permutation test was used to determine whether or not each metabotype was due to chance. A total of 1,000 random permutations of CAMP subjects were performed to test how frequently the diagnostic performance of a metabotype was observed in the random permutations. A metabotype test was considered valid (i.e., not considered to be a result found only by chance), if the combined diagnostic performance of at least 5% sensitivity, at least 95% specificity, and percent of ASD positive subjects in the metabotype-population at least 90% were met or exceeded with a frequency of 5% or less in the permutation test [Smith et al., 2019]. A metabotype test was considered to be reproducible if it also met the diagnostic performance and permutation test criteria required for the discovery set in the replication set. We made a strategic choice to maximize specificity in order to reduce the number of false positives associated with the combination of metabotype tests. Fewer false positives per metabotype test allow multiple tests to be combined into a test battery without significant loss of overall specificity.

As described below, we discovered a number of metabotypes associated with ASD in this study. Test batteries were generated by combining multiple metabotypes into a single test. If an individual was positive for any one of the metabotype tests within the test battery, it indicated that the individual is at higher risk for a diagnosis of ASD. In the current study, this test battery approach was used to determine the diagnostic performance of closely related tests within a metabotype cluster and for the development of an optimized screening test battery.

Results

Study Population

The CAMP study population was divided into two independent subject sets of children: (1) a discovery set of 357 subjects to establish metabotypes and (2) a

replication set of 351 subjects to establish the reproducibility of metabotypes and diagnostic performance (Table 1). The primary demographic values of age, sex, and DQ were balanced between discovery and replication sets. However, the percentage of male subjects, as well as age, and subject DQ differed between the ASD and TYP populations within the sets. The ASD population contained 17.3% and 22% more male subjects in the discovery and replication sets, respectively, which were 2.5 and 3 months older than the TYP populations. Due to the prevalence of co-occurring cognitive and developmental delays in the ASD population, the DQ was lower in the ASD group compared to the TYP population.

Differential Analysis of Metabolite Levels in ASD and TYP Subjects

Individual metabolites, and all unique combinations of the ratios of metabolites, were evaluated as potential screens for ASD. The ratios of metabolites were evaluated since this type of analysis can uncover biologically relevant changes not evident when evaluating each metabolite independently [Petersen et al., 2012; Smith et al., 2019]. When the metabolite and ratio values were adjusted for age, no differences in mean values were identified for the age, sex, or diagnosis of the subjects or their interactions (Table S2). Thus, the mean levels of the metabolites and their ratios are similar between ASD and TYP subjects regardless of age or sex. This indicates that demographic differences in age and sex between ASD and TYP populations are not likely to impact the conclusions of this study.

Metabotype-Based Test Development

Metabotype analysis of the discovery set identified 250 potential metabotype tests (Table S3) that met established diagnostic performance criteria. These tests were then evaluated in the replication set and 34 metabolite ratios reproducibly identified ASD metabotypes (Table S4). Among these 34, there were two that were previously reported [Smith et al., 2019], while the remaining 32 ratios were novel (Fig. 1, Table S4). Taken together, the 34 metabotypes identified 57% (95% confidence interval [CI], 52%–61%) of the CAMP ASD population with a total specificity of 83% (95% CI, 77%–88%).

Clusters of Metabotypes Identify Metabolically Distinct ASD Subpopulations

Correlation analysis and hierarchical clustering of the 34 reproducible amino acid and energy metabolism metabotypes were used to understand the relationships between the metabotype tests. We wanted to determine, for example, whether different metabotype tests identified the same groups of participants. We used hierarchical clustering (Fig. 2A) for the metabotype-positive subject population (Fig. 2B) to test for clusters of related metabotypes. Following bootstrap resampling analysis, the metabolite ratios formed six reproducible clusters of metabotype tests. Five of these clusters contain ratios that include one of the following metabolites: succinate, glycine, ornithine, 4-hydoxyproline, or α-ketoglutarate. A sixth cluster contains ratios that included either lactate or pyruvate (Fig. 2A).

Each of the clusters consists of several metabotype tests. Metabotype-positive subjects are generally identified by multiple metabotypes within the cluster (Fig. 2B). For example, numerous subjects within the lactate and pyruvate cluster (purple text) are positive for multiple metabotypes. The closer relationship of metabotypes within a cluster is also evident in the increased probability of being positive in more than one metabotype test within a cluster (Fig. 3). The newly identified metabotype clusters identify between 10% and 28% of the CAMP ASD population, with specificity greater than or equal to 95% (Table 2). The sensitivity of the clusters is greater than any of the individual metabotype tests within a cluster.

The succinate, 4-hydoxyproline, α-ketoglutarate, and lactate/pyruvate clusters identify novel metabotypes associated with ASD that have not been previously reported. The BCAA dysregulation metabotype (AADM) that we had previously described [Smith et al., 2019] identifies subpopulations of autistic individuals with elevated levels of the metabolites glycine, ornithine, or glutamine and lower levels of the BCAAs. The glycine and ornithine clusters reported here contain the AADM-associated metabolite ratios glycine/isoleucine and ornithine/leucine, respectively. These two clusters identify 70% of the subjects in the previously reported AADM metabotype-positive population (Fig. 2B) indicating that the metabotype tests in the glycine and ornithine clusters identify AADM metabotypes related to ornithine and leucine.

Association Analysis of ASD Subjects by Metabolic Cluster

The metabotype clusters were analyzed for associations with phenotypic information gathered on the ASD subjects related to medical history, behavioral testing, diet, supplements, and medications. Interestingly, the ornithine cluster identified a higher proportion of females (Fisher's exact test odds ratio 3.3 (95% CI, 1.83–6.00), FDR = 0.00068). The α-ketoglutarate cluster metabotype-positive subjects were more likely to be delivered by Cesarean section (Fisher's exact test odds ratio 2.23 (95% CI, 1.27–3.86), FDR = 0.044). The metabotype-positive population identified by the succinate cluster

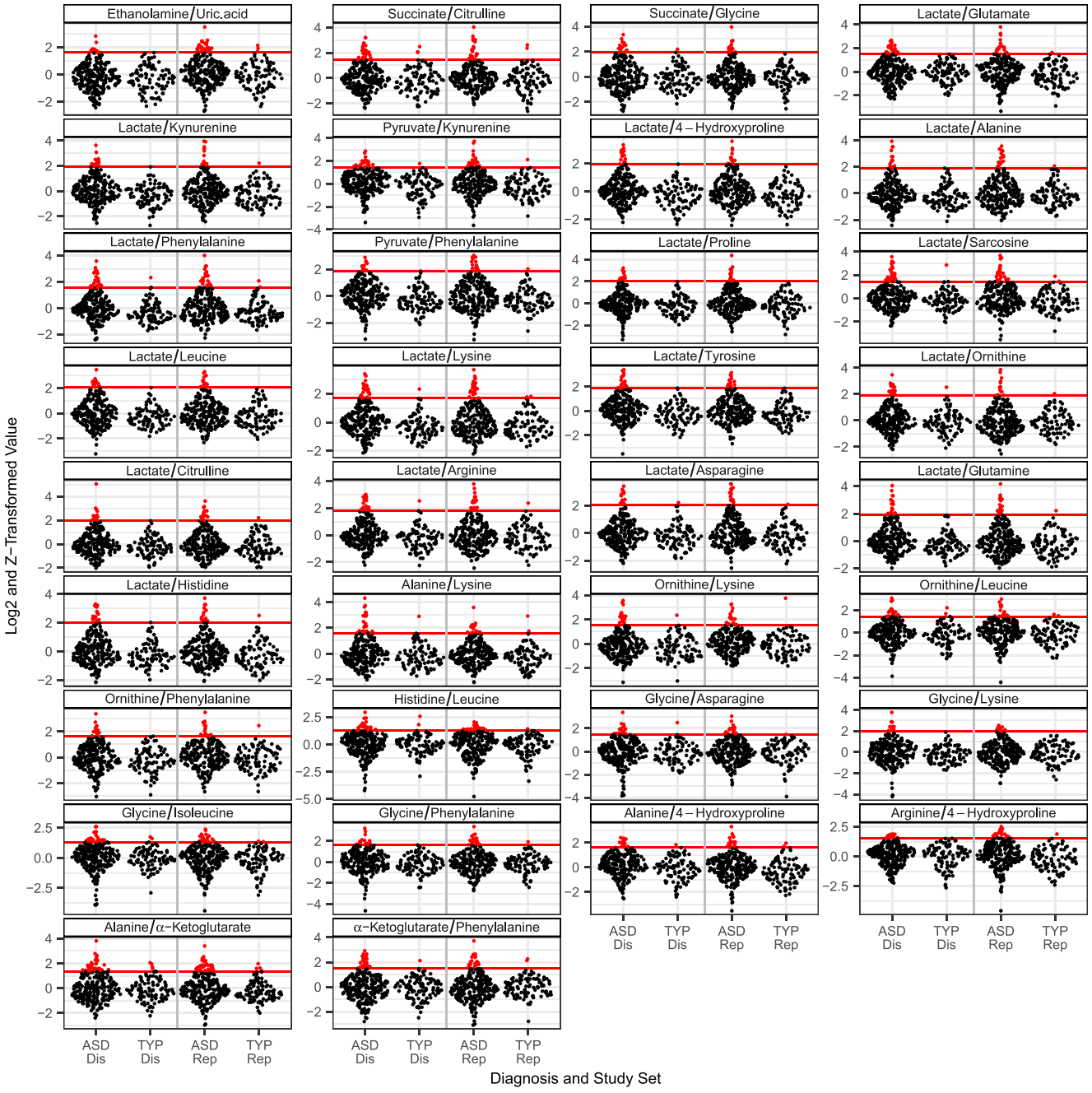

Figure 1. Scatter plots with distribution contours of the ratios measured in blood plasma for the 34 metabotype tests, meeting minimum diagnostic performance criteria. Metabotype-positive populations are generally composed of ASD subjects in both the discovery and replication sets. The positive subjects (red dots) are identified by the metabotype diagnostic threshold established in the discovery subject set (red horizontal line). The vertical gray line separates the discovery (on the left) from the replication (on the right) sets of subjects. The black dots are metabotype-negative subjects. The *y*-axis is log2 and then Z-transformed so that each ratio has a population mean of one and a standard deviation of zero. Distributions for the ASD and TYP populations are shown separately for each ratio and study set. Plots are ordered to be consistent with the dendrogram in Figure 2A. Dis, discovery set; Rep, replication set.

had 14% lower receptive language scores than the metabotype-negative population (–0.1432%; 95% CI, –0.229 to –0.057), T-test FDR = 0.024). Additional research will be needed to confirm and extend phenotypic characteristics of these metabotype clusters.

Optimized Metabotype Screening Test Battery

The fundamental goal of this research is to develop a metabolomics-based test battery that can be used as a screen for autism risk. As indicated above, the

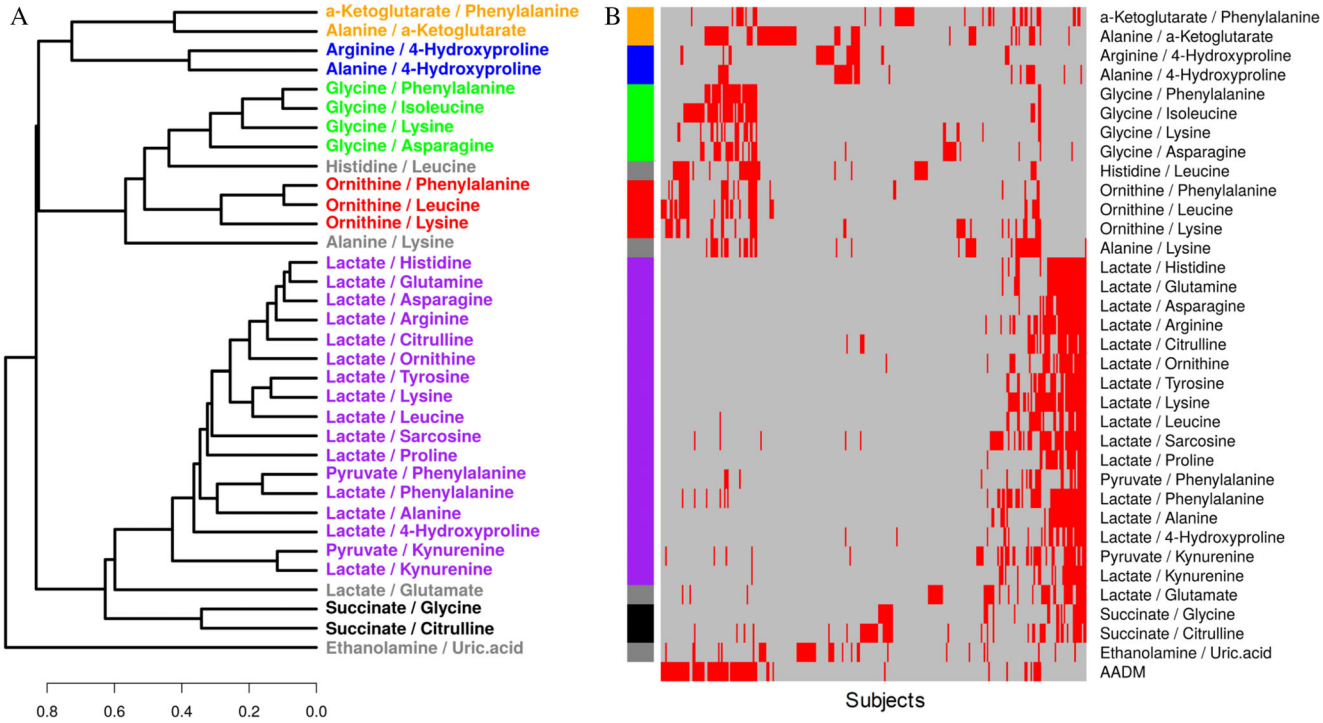

Figure 2. (A) Hierarchical clustering based on the pairwise Pearson correlation coefficients of the ratios of the 34 reproducible metabotypes. Bootstrap analysis identified six robust clusters of metabotype tests that are indicated by colored text associated with the dendrogram leaves (Table 2). The black text indicates the succinate cluster, purple text the lactate and pyruvate cluster, red text the ornithine cluster, green text the glycine cluster, blue text the 4-hydroxyproline cluster, and orange text the α-ketoglutarate cluster. The gray text indicates metabotypes not in one of the six clusters. The *y*-axis represents dissimilarity as a distance. (B) Heatmap of the metabotype-positive population. Individual subjects make up the columns of the figure. The 34 metabotypes are shown on the vertical axis as well as the BCAA dysregulation metabotype (AADM)-positive population [Smith et al., 2019]. The heatmap indicates that subjects are often positive for more than one metabotype and are often positive for more than one test in the same cluster. The AADM metabotype was included to highlight the similarity of the glycine and ornithine ratios to the previous findings. The rows of the heatmap and colored blocks are to highlight the metabotype groups in (A). Red represents metabotype-positive and gray represents metabotype-negative subjects.

metabotype tests within each cluster redundantly identified a similar group of ASD subjects Figure 3, Tables S5 and S6), We sought to create an optimized test battery based on selecting a subset of the 32 novel metabotype tests that (1) maximized sensitivity while maintaining a specificity of at least 90% to provide more diagnostic value to a positive test result, (2) contained at least one metabotype test from each of the six clusters to represent biological information from each cluster in the final test battery, and (3) eliminated redundant tests. To reduce the number of redundant tests, a subset of tests from each cluster was selected that identified the ASD participants detected by all of the tests within a cluster. This process led to the selection of 19 metabotype tests that captured the total sensitivity identified by each of the clusters. We then created test batteries containing 7–18 metabotype tests using combinations of the 19 tests. The test combinations were filtered by diagnostic performance in the combined discovery and replication sets. The maximum observed sensitivity of test combinations was 50% at specificities of at least 90%. The optimal combination selected for the final test battery contained 14 metabotype tests that represented each cluster and yielded the highest sensitivity in the discovery and replication sets with a specificity greater than 90% (Fig. 3). This optimized test battery identified CAMP subjects with a sensitivity of 50% (95% CI, 45%–54%) and specificity of 92% (95% CI, 88%–96%). Addition of the AADMs test predictions to the optimized test battery increased the overall sensitivity to 53% (95% CI, 48%–57%) with a specificity of 91% (95% CI 86%–94%). When compared to the diagnostic performance of the combination of the 34 metabotype tests, the optimization process led to a reduction in the number of tests, and importantly, to the reduction of false positives, thereby increasing the specificity by 8%. Total sensitivity was reduced from 57% to 53% due to the elimination of tests that contributed an unacceptable number of false-positive results to the overall test battery.

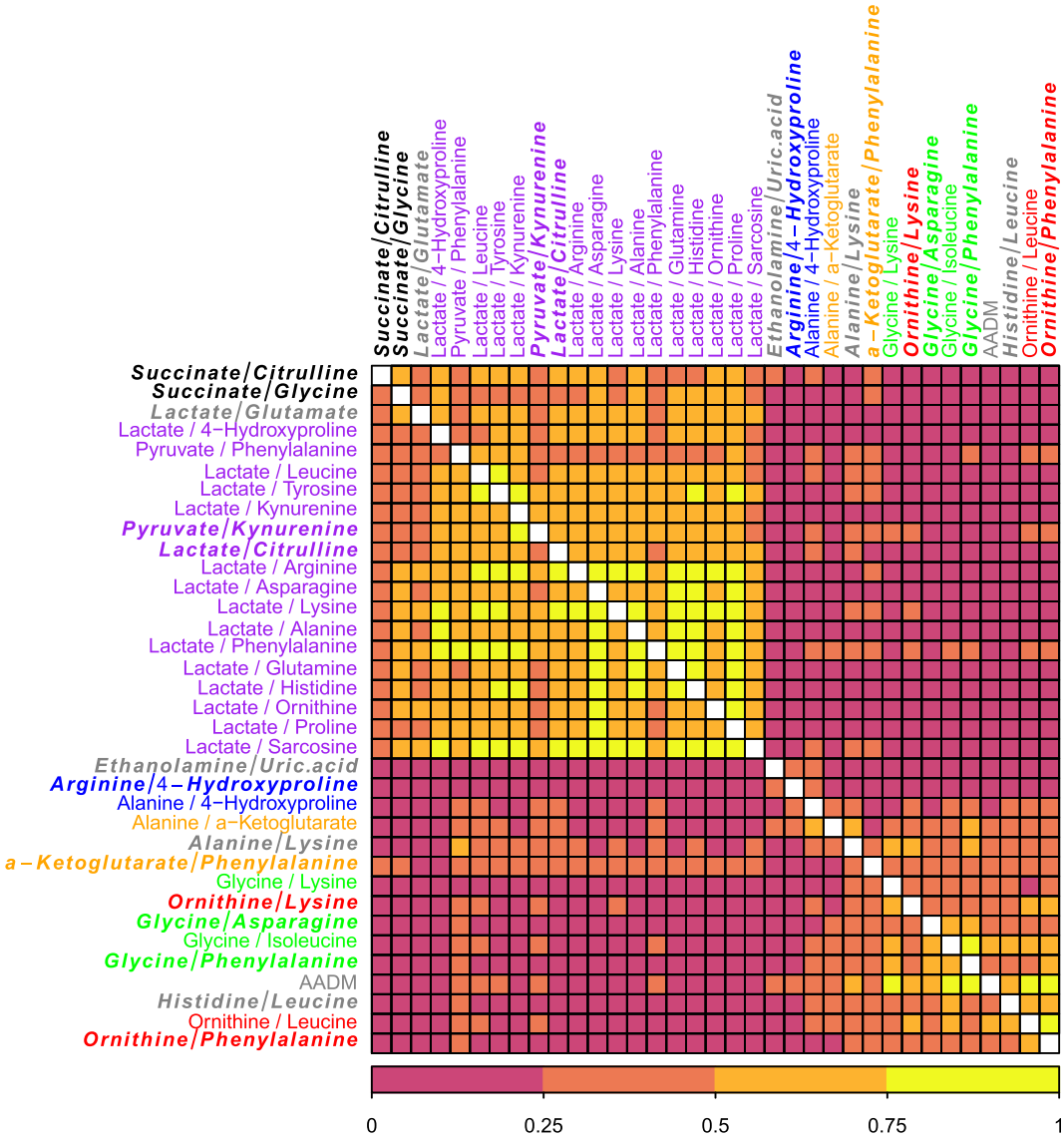

Figure 3. Heatmap of the similarity of metabotype test subject predictions based on the conditional probability of a subject testing positive for the metabotype in the row given testing positive for the metabotype in the column. The analysis provides visualization for how frequently subjects test positive for one test given that they are positive for another and further supports that clusters of the plasma values are largely mirrored in the metabotype predictions. The conditional probabilities are also helpful in reducing the overall number of tests required to identify metabotype-positive subjects within a cluster. The color scale indicates the conditional probability that a subject will test positive for the metabotype in the row given a positive result in column test. Tests are ordered using hierarchical clustering to simplify the visualization. Colored text associated with the column and row labels indicate the six clusters identified in Figure 2A. The black text indicates the succinate cluster, purple text the lactate and pyruvate cluster, red text the ornithine cluster, green text the glycine cluster, blue text the 4-hydroxyproline cluster, and orange text the α-ketoglutarate cluster. The gray text indicates metabotypes not in one of the six clusters. BCAA dysregulation metabotype (AADM)-positive population is included. Bold and italicized leaves denote ratios used in the optimized test battery.

Discussion

The CAMP study was designed to reproducibly identify subpopulations of autistic children as small at 5% who share common metabolic differences from TYP children (i.e., metabotypes). The study involved 499 children that had a diagnosis of ASD and 208 children that were TYP and were able to contribute blood samples that met quality control standards for metabolic analyses. We quantitatively measured 39 metabolites associated with amino acid and energy metabolism. This set of metabolites was initially chosen for analysis based on pilot studies [Smith

Table 2. Diagnostic Performance of the Metabotype Clusters

Metric	Subject set	4-HP	α-KG	Gly	LacPyr	Orn	Other	Suc
Sensitivity (95% CI)	Discovery	0.10 (0.06–0.14)	0.23 (0.18–0.29)	0.15 (0.11–0.2)	0.19 (0.14–0.24)	0.12 (0.08–0.17)	0.28 (0.22–0.34)	0.12 (0.08–0.17)
	Replication	0.13 (0.09–0.17)	0.22 (0.17–0.28)	0.14 (0.1–0.19)	0.18 (0.13–0.23)	0.13 (0.09–0.18)	0.31 (0.25–0.37)	0.11 (0.08–0.16)
Specificity (95% CI)	Discovery	0.99 (0.95–1.00)	0.95 (0.89–0.98)	0.98 (0.93–1.00)	0.97 (0.92–0.99)	0.98 (0.93–1.00)	0.98 (0.93–1.00)	0.97 (0.92–0.99)
	Replication	0.97 (0.92–0.99)	0.94 (0.88–0.98)	0.97 (0.92–0.99)	0.93 (0.87–0.97)	0.98 (0.93–1.00)	0.94 (0.88–0.98)	0.98 (0.93–1.00)

Note. Abbreviations of clusters: α-KG, α-ketoglutarate; 4-HP, 4-hydroxyproline; Gly, glycine; LacPyr, lactate or pyruvate; Orn, ornithine; Other, metabolites not contained in a cluster; Suc, succinate.

et al., 2019; West et al., 2014] and published research related to abnormalities of purine metabolism and mitochondrial bioenergetics [Broek et al., 2014; De Rubeis et al., 2014; László et al., 1994; Shen et al., 2019; Yehia et al., 2019]. We observed that (1) analysis of ratios of plasma metabolite concentrations revealed 34 metabotype tests that reproducibly identified metabolic differences associated with ASD and (2) these metabotypes formed six distinct clusters related to the underlying metabolic dysregulation. A battery of 14 metabotype tests, when integrated with previously identified metabotypes [Smith et al., 2019], identified ASD subjects within CAMP with a sensitivity of 53% (95% CI, 48%–57%) and a specificity of 91% (95% CI 86–94%).

Our Strategy for Metabotype Analysis

There has been intense interest in discovering effective and practical metabolite assays for the identification of children at risk for ASD. Disappointingly, most previously described "diagnostic tests" have generally not been reproduced in subsequent studies. Lack of reproducibility is likely due to several issues including the etiological and phenotypic heterogeneity of ASD, and the small number of cases vs controls in most previous studies [Loth et al., 2017]. Our metabotyping approach starts from the premise that different subgroups of individuals with autism will have different metabolic signatures. Our analytic approach quantitatively explores domains of metabolites to find those that identify homogenous subpopulations of individuals with ASD. We explicitly do not attempt to create a single, broad-based predictive signature of ASD, i.e., we acknowledge the heterogeneity of ASD. Moreover, the size of the CAMP study population provides sufficient power to enable both a discovery and an independent replication set of subjects each larger than the total number of subjects in most previously published metabolism studies of autism.

The autism literature provides clues to which metabolic anomalies should be investigated. However, the design attributes of this study (e.g., large cohort size with replication set, validated analytical methods, and subtyping approaches) allow for a significant extension of prior work. For example, altered metabolism among individuals with ASD has been observed related to biochemical pathways including oxidative phosphorylation and BCAA metabolism [Glinton & Elsea, 2019; Hollis, Kanellopoulos, & Bagni, 2017]. The current work draws from the earlier studies to reproducibly identify and stratify metabolic alterations common in specific groups of subjects such that they can be used to begin further work toward therapies that are specific to defined metabotypes.

Ratios of metabolites can increase diagnostic efficacy by detecting metabolic associations and biochemical pathways not apparent in the analysis of single

metabolites [Petersen et al., 2012]. For example, metabolite ratios of bloodspot-derived amino acids and acylcarnitines have been successfully used in newborn screening for metabolic disorders such as phenylketonuria, maple syrup urine disease, and certain disorders of mitochondrial fatty acid beta-oxidation [Arneth & Hintz, 2017; McHugh et al., 2011]. Prenatal serum metabolite ratios can predict fetal growth restriction [Sovio et al., 2020]. In view of the value of metabolite ratio analysis, we analyzed all possible combinations of the 39 plasma metabolite pairs related to amino acid, purine catabolism, and energy metabolism in a supervised approach to identify potential metabolic subpopulations associated with ASD. Whereas none of the levels of individual metabolites met the diagnostic criteria required in the discovery set, ratios of these metabolites led to 34 metabotype tests that reproducibly identified metabotypes.

Alterations in Metabolite Ratios May Provide Insight into Pathophysiology

While the primary goal of this research program is to establish reliable metabolomic screens, a related aim is to provide insight into metabolic disturbances that may lead to more targeted treatments. Hierarchical clustering of metabotypes established six clusters of metabotype tests related to amino acid and mitochondrial energy metabolism. The metabolic clusters are comprised of ratios containing (1) lactate or pyruvate, (2) succinate, (3) α-ketoglutarate, (4) glycine, (5) ornithine, and (6) 4-hydroxyproline in combination with other metabolites. These clusters highlight potential dysregulation in amino acid and energy metabolism in ASD when compared to TYP. It is important to point out that the dysregulation that we report occurs at quantitative metabolite levels that for any of the studied metabolites are not diagnostic of specific clinical disorders. But, when evaluated as ratios, they identify changes that are outside the normal range of values observed in the vast majority of TYP children.

Alterations in succinate, lactate, and pyruvate concentrations and their ratios are often associated with disturbances of mitochondrial bioenergetics [Lombard, 1998; Shaham et al., 2010; Thompson Legault et al., 2015; Vergano et al., 2014], and these disturbances occur with increased frequency in people with ASD [Hollis et al., 2017; Rose et al., 2018; Rossignol & Frye, 2012; Weissman et al., 2008]. The overlap of ASD subjects identified by metabotype tests in the lactate/pyruvate cluster suggests that they may all experience similar dysregulation and underlying pathophysiology. While one might expect that the succinate and α-ketoglutarate clusters would be closely related to the lactate and pyruvate cluster as intermediates of the tricarboxylic acid (TCA) or Krebs cycle, they actually identify largely different subsets of ASD cases. Subjects identified by the α-ketoglutarate cluster were only infrequently positive in the succinate (10%) or pyruvate and lactate (29%) clusters (Table S6). This raises the possibility that these two groups of autistic individuals have different underlying pathophysiologies (Fig. 4). We hypothesize that this may be due to complex biological roles illustrated in Figure 3 that succinate and α-ketoglutarate play in signaling outside the TCA cycle [He et al., 2015; Murphy & O'Neill, 2018].

Metabotype-positive ASD participants in clusters containing ornithine, glycine, α-ketoglutarate, and 4-hydroxyproline are mostly (67%–94%) metabotype negative for ratios containing succinate, lactate, or pyruvate (Table S6), again suggesting differences in the underlying metabolism of these two groups. The metabotype ratios fall into two larger clusters, one comprised of ratios containing α-ketoglutarate, glycine, ornithine, and 4-hydroxyproline and a second containing ratios with lactate, pyruvate, and succinate. The metabotype-positive subjects in the first group of clusters may be related to dysregulation of amino acid metabolism and the urea cycle (Fig. 4), while metabotype-positive participants in the second group of clusters may have dysregulation related to energy metabolism or mitochondrial function. Furthermore, the ASD participants who are metabotype-positive for ornithine and glycine clusters are very similar to the previously described AADM metabotype population [Smith et al., 2019] with increased ornithine and glycine and decreased levels of BCAAs. Individuals who are metabotype-positive for 4-hydroxyproline do not have much overlap with those who are metabotype-positive for the ornithine, glycine, or AADM populations, and are more similar to the α-ketoglutarate cluster. Thus, the six clusters of metabotype tests that we have discovered highlight a diversity of underlying metabolic alterations. Although the pathophysiological basis of these alterations is not understood at this time, our approach provides a stratification mechanism to facilitate research into the underlying biology related to each of these metabotypes.

Are There Functional Associations of the Metabotypes?

Analysis of phenotypic data of the autistic subjects revealed some intriguing, albeit very preliminary, associations between subjects with a certain metabotype and biological or behavioral features of the ASD cohort. For example, there was an overrepresentation of female subjects identified by the ornithine-related metabotypes. Ornithine aminotransferase, ornithine decarboxylase, and arginase-2 are regulated by testosterone [Levillain, Diaz, Blanchard, & Déchaud, 2005], which could explain sex-specific differences observed in ASD [Ferri, Abel, & Brodkin, 2018]. Interestingly, subjects in the α-ketoglutarate metabotype-positive cluster were more likely to have had a Cesarean delivery (CD). Children born by CD

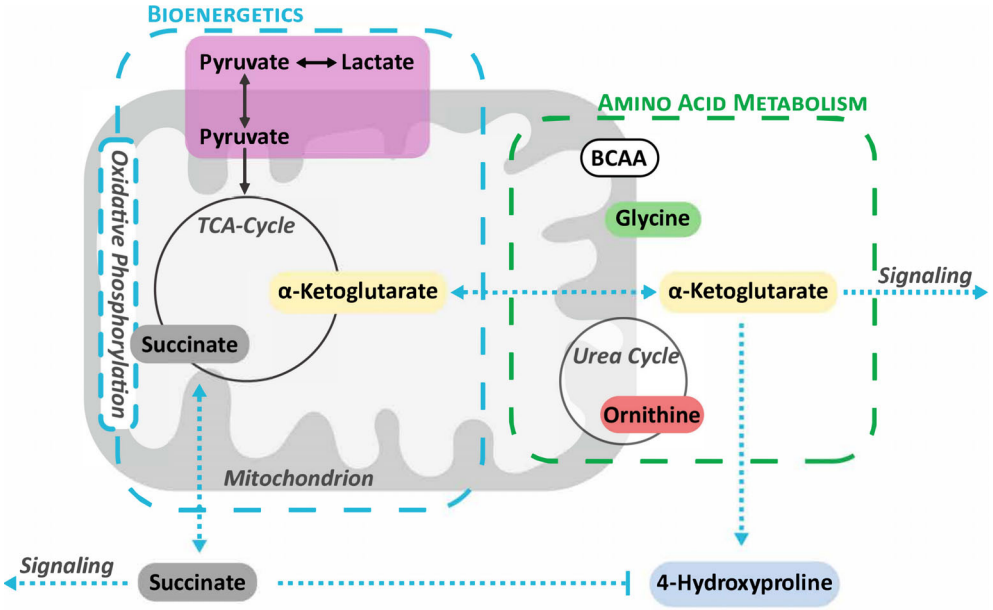

Figure 4. Representation of identified metabotype clusters and their biological interconnectivity. Boxes are colored according to reproducible clusters in Figure 2A. The metabolites associated with the clusters participate in many metabolic pathways and signaling processes. Pyruvate is a "crossroads" metabolite at the juncture of glycolysis, gluconeogenesis, and the TCA cycle. It represents the main gateway to convert glucose to energy in mitochondria. Lactate, the reduced product of pyruvate, is itself a potential energy substrate. Some metabolites (e.g., α-ketoglutarate and succinate) form distinct metabotype clusters, likely reflecting different underlying pathophysiologies, despite being biochemically connected. Succinate and α-ketoglutarate are intermediates in the TCA cycle and donate electrons to the electron transport chain to generate energy through oxidative phosphorylation. Yet, succinate and α-ketoglutarate also have important additional roles outside of the TCA cycle and oxidative phosphorylation. Thus, clusters may identify distinct metabotype populations based on their roles in signaling processes rather than the TCA cycle or oxidative phosphorylation. Additionally, α-ketoglutarate, glycine, the BCAAs, and the urea cycle metabolite ornithine play important roles in amino acid and nitrogen metabolism. The interconnectivity of metabolic and signaling processes can explain why some patients might be positive for metabotypes from different metabolic pathways while seemingly biochemically related metabolites can form distinct metabotype clusters.

tend to have an increased body mass index [Bernardi et al., 2015], altered microbiome, and immune function [Liu et al., 2018; Wampach et al., 2018], each of which is associated with increased risk of ASD [Healy, Aigner, & Haegele, 2019; Vuong & Hsiao, 2017; Zhang et al., 2019]. Lastly, subjects in the succinate cluster had decreased receptive language scores compared to metabotype-negative subjects. These preliminary observations need to be replicated and extended in future studies, but they highlight the potential that subtle, yet reliable, metabolic alterations may be associated with functional outcomes.

An Approach to Developing a Metabolomic Screening Platform for ASD

The results reported in this article are another step toward developing a platform of metabolic tests that can be used to determine relative risk for an individual to have a diagnosis of autism. Such a platform could increase effectiveness of screening efforts by providing additional decision-making end points [McPartland, 2017; Zwaigenbaum et al., 2015]. Developing a metabolomics-based screening tool is an ongoing process. As we define additional metabotypes from samples acquired through the CAMP study, the percentage of ASD subjects that can be identified will increase. Currently, the optimized metabotype test battery is capable of identifying 53% of the 18- to 48-month-old CAMP ASD participants with a specificity of 91%. The implication of these results, which must be verified in a prospective study, is that this metabolomics-based test battery is potentially able to detect more than 50% of individuals at risk for ASD. While biomarkers of any kind cannot provide a definitive diagnosis, combining a metabolomics-based screen with a behavioral screening tool such as the M-CHAT/F increases the likelihood that those at risk for ASD can be detected as early as possible [Kohane & Eran, 2013; Miles, 2015].

How Would a Metabolomics-Based Screen Be Deployed?

Metabotype-based tests can support earlier diagnosis by identifying subsets of children having metabolic differences associated with ASD. In practice, we envision a metabolomic-based test as both an additional screening

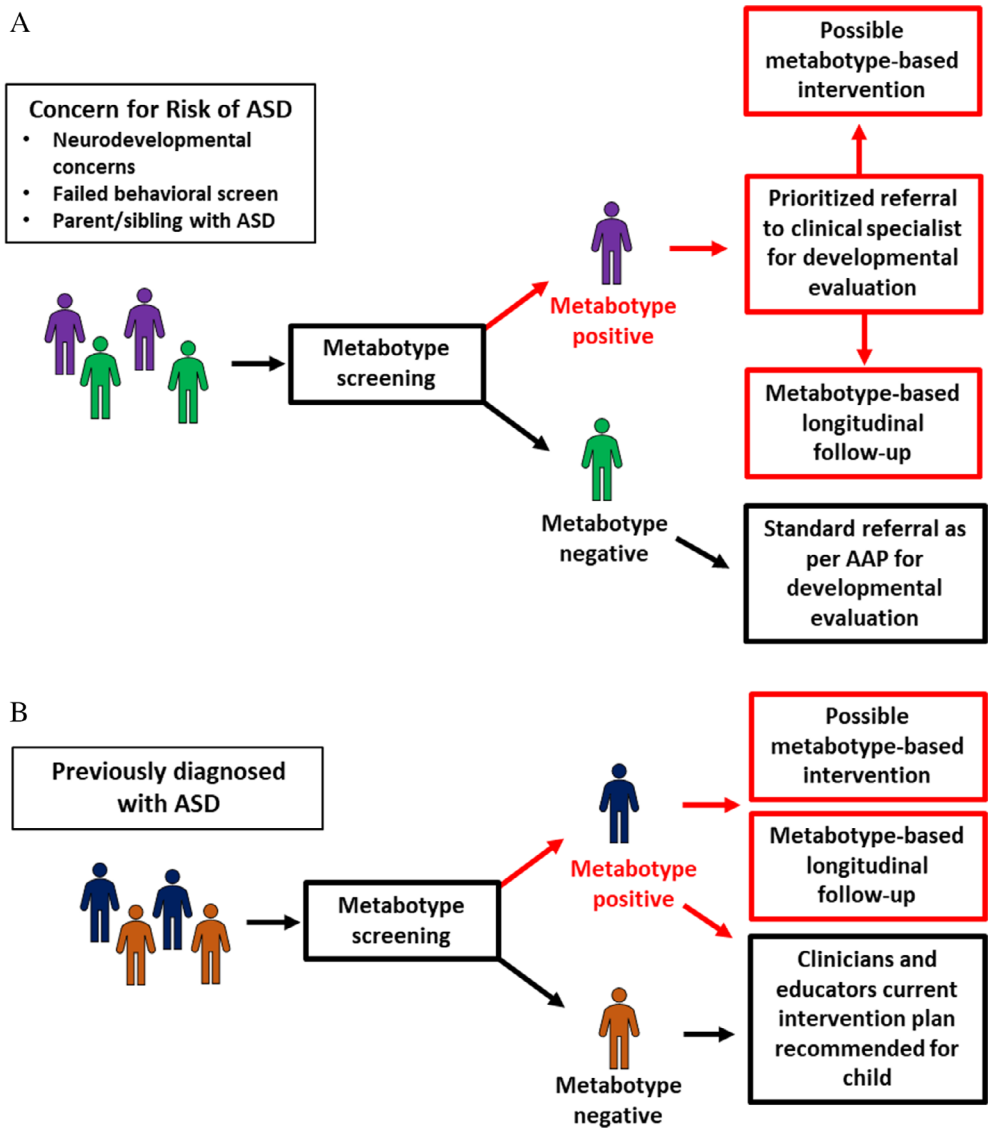

Figure 5. Proposed applications for metabotype-based screening and potential outcomes for metabotype-positive and metabotype-negative children at risk of ASD (A) and children previously diagnosed with ASD (B). See text for description.

modality to detect children who are at risk for a diagnosis of ASD and as a stratification tool (Fig. 5) for individuals who are already diagnosed. A child for whom there may be grounds for evaluation based on family history or because of clinical or parental concerns would be a candidate for metabotype-based screening. A positive metabotype result could lead to a prioritized referral to a specialist for diagnostic assessment of ASD. A metabotype-negative result would follow the American Academy of Pediatrics (AAP) standard of care for further behavioral and developmental assessment at periodic intervals in early childhood (Fig. 5A). Individuals already diagnosed with ASD may benefit in the future from metabotype screening for insight into metabolic dysregulation that could potentially lead to a refined, personalized intervention plan (Fig. 5B).

Additional Considerations and Some Limitations of This Work

Several issues related to this work require additional research. These include: (1) individuals with other neurodevelopmental disorders were not included in the study so it is unclear how selective the metabotypes are for ASD; (2) only a portion of the plasma metabolome was evaluated and further metabolomic analyses are needed to discover additional metabotypes; and (3) the lack of a broader age range and of longitudinal sampling

prevents assessment of metabotype utilization and stability for ages other than those evaluated in this study. This study also has several limitations: (1) the study size does not allow us to detect metabotype populations that occur in less than 5% of the ASD population, so rare metabolic dysregulation may not be detected by this approach and (2) analysis of fasting plasma samples may miss metabolic dysregulation that can be of clinical relevance but whose discovery may require other types of samples like those obtained after physiological challenges such as glucose or lipid loads.

Conclusions

The CAMP study has produced a unique repository of samples from children with autism and age-matched TYP controls that will enable an ongoing exploration of small molecule signatures of risk for ASD. Our first study, which focused on BCAA metabolism, enabled the detection of 17% of the CAMP ASD cohort [Smith et al., 2019]. The current study, which focused on 39 metabolites associated with amino acid and energy metabolism, has enabled the detection of 50% of the autistic subjects. Taken together, the current test battery can detect 53% of the children with ASD in CAMP. The future direction of this program is to uncover additional metabotypes which will increase even further the detection percentage of children at risk for a diagnosis of ASD. Many important areas of research remain. It is not clear, for example, how stable these metabotypes are in a particular child. If we brought the CAMP cohort back when they are in middle childhood, would they demonstrate the same metabotypes? Would we see the same metabotypes in children younger than 18 months when the value of a biomarker would be greatest? Given the virtual absence of effective biomarkers to detect autism risk in young children, we are optimistic that this approach has enormous potential for identifying children as early as possible. Moreover, determining that an individual child has a particular pattern of metabolic alterations can provide insights into her or his autism and offer the possibility of new personalized therapies. These opportunities and questions require further testing in CAMP and future studies. These steps represent strides in a research journey toward understanding the role of metabotypes and their potential as actionable clinical tools in the detection and treatment of ASD.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Chemical Reference Compounds and Ions Measured by Mass Spectrometry Methods.

Table S2. Analysis of Covariance of Diagnosis and Sex Controlling for Age in the Discovery and Replication Sets.

Table S3. 250 Metabotype Tests Meeting Minimum Performance Metrics in the Discovery Set of Subjects.

Table S4. 34 Metabolite Ratios That Identify Metabotypes of Autism Spectrum Disorder Meeting Minimum Performance Criteria in Both Discovery and Replication Sets.

Table S5. Similarity of Metabotype Ratio Tests Based on Conditional Probability of Metabotype Positive Results.

Table S6. Similarity of Metabotype Clusters Based on Conditional Probability of Metabotype Positive Results.

Table S7. Elution Gradient for the Amine Liquid Chromatography Method.

Table S8. Elution Gradient for the Purine Liquid Chromatography Method.

Table S9. Elution Gradient for the Energy Liquid Chromatography Method.

Appendix S1: Supporting Methods.