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Precision medicine approaches for heterogeneous conditions such as autism spectrum disorders (the need for a biomarker exploration phase in clinical trials - phase 2m)

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Editorial: Precision medicine approaches for heterogeneous conditions such as autism spectrum disorders (The need for a biomarker exploration phase in clinical trials - Phase 2m)

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Editorial on the Research Topic
Precision medicine approaches for heterogeneous conditions such as autism spectrum disorders (The need for a biomarker exploration phase in clinical trials - Phase 2m)

Significant progress has been made in understanding the biology of autism spectrum disorder (ASD), providing rational hypotheses for interventions to address the core symptoms. However, clinical trials of these interventions have failed to yield positive results to date. In many of these studies, a subset of participants appear to respond well, but a significant benefit is not found in the overall intent-to-treat group. Due to the etiological heterogeneity of ASD, we anticipate that this will continue to be a challenge in future clinical trials. It will be critical to identify the patients that are most likely to respond to a treatment and to target those subjects in later phase trials. We

therefore propose the explicit inclusion of "Phase 2m" as part of the pathway of clinical drug development, specifically for the development of a biomarker profile that can be incorporated into later phase 2 and 3 clinical trials. Such a precision medicine approach has the potential to optimize the likelihood of success in future clinical trials to benefit patients.

Introduction

Autism spectrum disorder (ASD) is highly heterogeneous, with estimates of potentially 1,000 genes that may be associated with risk for ASD (1). This is in addition to cases with environmental and other non-genetic contributors such as infection and inflammation during pregnancy (2), as well as cases that would at this point be considered idiopathic cases. Studies using cellular and animal models have pointed to underlying neurobiological systems and pathways impacted by individual ASD risk genes, with some suggestion of convergence across genes. For example, research exploring the synaptic mechanisms impacted by the fragile X syndrome (FXS) gene *FMR1* led to trials with negative allosteric modulators of metabotropic glutamate type 5 receptors in FXS (3). Converging lines of evidence (4, 5) led to clinical trials that target glutamatergic and GABAergic functions in ASD; however, these studies have failed to yield positive results for primary outcome measures. The GABA-B receptor agonist arbaclofen, for example, did not show significant benefit on its primary outcome measure in a phase 2 clinical trial for ASD (6). The high degree of heterogeneity within ASD likely contributes to these failures, as a treatment designed to target one biological etiology of ASD may not have a beneficial effect on patients with ASD resulting from perturbations in other biological pathways.

Heterogeneity and biomarkers in ASD

Heterogeneity in ASD can be observed in multiple dimensions, from core symptom pattern to cognitive or communication ability to identifiable risk factors. Genetics has been proposed as a method of subtyping autism (7–17). Rare variants with high penetrance that are directly involved in the etiology of ASD have provided major insights for development of novel therapeutics, while other genes serve as risk factors for ASD that may act in concert with other genetic or environmental risk factors (1, 7–10). However, therapeutics designed to target one specific etiology have an unknown impact on other etiologies of ASD. Additionally, there is a need for greater understanding of pleiotropy within each specific etiology, whereby one might respond to a specific treatment but not another within this group.

In hopes of examining common downstream pathways of the effects of individual etiologies on neural systems, other biomarkers have been assessed in ASD, including markers of brain structure and activity (EEG, imaging) (18–27). A recent study by Ellegood et al. found that 26 different ASD-associated mouse models converged onto three clusters of brain anatomical features from MRI (28). This suggests that neuroimaging may be a powerful tool in the identification of ASD subtypes with specific treatment response, despite genetic heterogeneity; although cost and feasibility issues may limit neuroimaging, particularly in young and more impaired ASD patients. Other types of biomarkers that may be helpful include epigenetic (29, 30), transcriptomic (31–33) (Beversdorf et al.), proteomic (34), and metabolomic markers (35, 36), as well as neurobehavioral measures such as eye-tracking and pupillometry (37–40), actigraphy (41), and psychophysical measures (42). The presence or absence of co-occurring medical (seizures, sleep disturbances, gastrointestinal conditions) and psychiatric conditions (aggression, anxiety, attentional deficits) also contributes to heterogeneity and certainly impacts the approach to treatment.

Heterogeneity is also seen in the core domains of ASD, including social communication and reciprocity deficits, repetitive behaviors/hyperfocused interests, and sensory symptoms. With such disparate symptoms, it may be difficult to formulate ASD severity along a single dimension or to model this unitary diagnosis in epidemiological research or in animal models. The Research Domain Criteria (RDoC) initiative at the National Institute for Mental Health (43) focuses on specific behavioral or cognitive domains within psychiatric or neurodevelopmental diagnoses and may be more tractable for research that spans methods. In support of this, data-driven brain imaging studies have found that brain networks contribute to social communication in a manner that is not diagnosis specific (44). Furthermore, recent studies of the structure of ASD symptoms have suggested four or more distinct social communication dimensions and five separate restricted/repetitive behavior subdomains (45–49). Targeting specific symptom domains would seem advantageous for such a heterogeneous condition as ASD. Recognizing heterogeneity across multiple dimensions, however, it is possible that an intervention may benefit a specific symptom domain in one specific etiology of ASD, with uncertainty about whether this will extend to the broader group of individuals with ASD diagnoses (2).

Within the heterogeneity of ASD, some biomarkers may predict a subpopulation with common disease mechanisms and may therefore be predictive of treatment efficacy. As one of the few examples of the potential utility of biomarkers to dissect heterogeneity within ASD treatment studies, low baseline plasma oxytocin level predicted response to intranasal oxytocin for social responsivity; although this did not replicate in a larger study (50). There are other obvious opportunities

to tap into this approach. Alterations in the glutamatergic and GABAergic systems are found with some consistency in ASD postmortem brain studies (51–53), as well as *in vivo* with magnetic resonance spectroscopy (MRS), albeit with some variability across brain regions (54–56). Some large clinical trials for core symptoms of ASD targeted glutamatergic (memantine) and GABAergic (arbaclofen) systems (6). It is possible that direct or indirect markers of GABAergic or glutamatergic system activity, such as MRS (57) (Nair et al.) or EEG gamma band activity (58), would have been valuable in predicting response in a subgroup of individuals, recognizing that no significant effect was seen in the overall group of participants with ASD. Whole blood serotonin (59, 60) or serotonin receptor binding on positron emission tomography (61–65) could similarly predict responses to treatments targeting serotonergic pathways (66). Psychophysical reactivity indicative of sympathetic/parasympathetic tone (67) could identify subjects that may be more responsive to adrenergic treatments (68). In other cases, we may not have obvious biomarker candidates to parse the heterogeneity in ASD treatment studies.

Additionally, the developmental trajectory must be considered in any approach, as mechanisms of actions that impact the developmental trajectory of neural systems at one stage may have an entirely different relevance at a later stage (69). Among the well-replicated imaging findings in ASD is anatomical overgrowth in the first post-natal years (70–74), and some continue to have larger heads later in life resulting from this (75, 76). It would seem that administration of an agent affecting growth trajectories would have remarkably different effects at different ages. Additionally, the impact of the developmental trajectory is likely critical for a wide variety of other factors as well. Thus, temporal factors must also be accounted for in the heterogeneity of ASD to best facilitate individualized treatment approaches and to move toward personalized medicine in ASD.

Incorporation of a biomarker exploration phase (phase 2m) in clinical trials

The incorporation of rich biobehavioral data to allow subgrouping of participants in clinical trials has the potential to identify which subjects are most likely to respond to a given treatment, and which clinical signs or symptoms are most responsive to that treatment (2, 77, 78). However, the current template of phases for drug development does not regularly incorporate this. In clinical drug development, **phase 1** trials are "dose escalation" or "experimental medicine" trials, focused on the safety and tolerability of drugs, and pharmacokinetics and pharmacodynamics are also assessed. These are followed by **phase 2** trials, where the findings of the first phase are harnessed for further safety, pharmacokinetic, and pharmacodynamic assessment with optimization of dosing and endpoints to be targeted in subsequent phases. **Phase 3** is the confirmatory therapeutic trial, or pivotal trial, conducted in a double blinded manner in a larger population, with statistical power to achieve the predetermined target outcomes based on the phase 2 findings. Successful phase 3 trials are followed by drug approval and marketing, with subsequent **phase 4** studies using observational monitoring to evaluate adverse reactions too infrequent to be detected in phase 3, for monitoring clinical efficacy in the broader population, and to assess cost effectiveness (79). Given the heterogeneity in ASD, it is unreasonable to expect any drug to benefit the majority of individuals, but ASD clinical trials have not had sufficient sample sizes to detect improvement in a subset. While the pharmacodynamic aspect of Phase 1 and 2 trials might be used to identify useful biomarkers and precision medicine targets, this has not commonly been the case for autism drug development. Not surprisingly, then, drug development programs in ASD have typically failed in phase 2 or 3.

A strategy, therefore, must be implemented early in the clinical trial pathway (Figure 1), for identifying biomarkers that can facilitate and inform future trials of the drug in development. In light of the failures of recent large ASD trials (5), we propose that early in Phase 2, a study or studies that could be considered as **phase 2m** (marker exploration phase) should include a rich set of biomarkers that are assessed in a moderately large population of participants to gain an understanding of which subjects respond best, thereby informing the final design and statistical power of later phase 2 and 3 trials. To maximize the richness of the biomarker monitoring, it would be tempting to use a design where all patients will receive the drug, however an open label design is at risk of identifying biomarkers that predict spurious (placebo-related or spurious) response. Blinded crossover designs or staggered start designs might be an appropriate alternative. The participants' developmental stage would also need to be considered as critical marker in this phase. Some markers might be mechanistic, such as biomarkers of GABAergic activity that could predict response to GABAergic agents in ASD. A broader biomarker profiling approach that spans phenotypic subgroups whose mechanistic basis or effects are not fully understood, such as macrocephaly, hyperserotonemia, or elevated IL-6, would better allow the matching of treatments with biomarkers that were not be predicted *a priori*. Other critical questions that could also be addressed include whether earlier intervention could lead to improvement not only in symptoms at the time of the trial but also an improved developmental trajectory. Thus, age of participation and long-term follow-up may be other crucial components to consider for incorporation in future clinical trials.

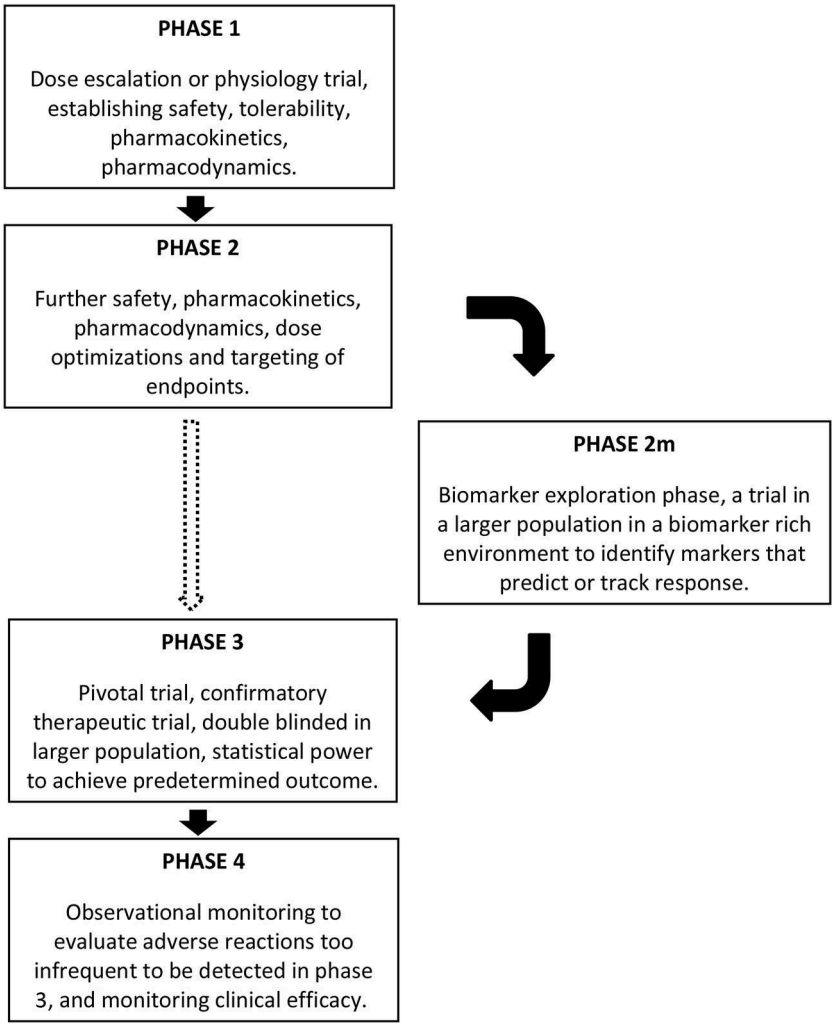

FIGURE 1
Schematic representing the location of a phase 2m in clinical trials, after phase 1 and phase 2, and before phase 3, if the need for a biomarker exploration phase is warranted based on the nature of the clinical condition. The dashed arrow moving directly from phase 2 to phase 3 might be appropriate for some conditions, but incorporating the phase 2m seems warranted in conditions such as ASD.

Conclusions

With our improving understanding of the genetic and environmental etiologies of ASD and the effects on specific neural systems during distinct developmental epochs, this information can be used for optimization of future clinical trials. By incorporating studies that focus on the predictive value of baseline biomarkers, while also exploring biomarkers that change with treatment and may index response, we can improve the likelihood of success in **phase 3** clinical trials. Integrated approaches to better understand the heterogeneity of autism have been initiated by large collaborations that include clinical trials, such as the Autism Innovative Medicines Study–2-Trials (AIMS-2-Trials) (80–82), as well as the Province of Ontario Neurodevelopmental Disorders (POND) Network (83). Additionally, recent work in the Autism Biomarker Consortium for Clinical Trials (ABC-CT) has been developing neurobehavioral markers, including EEG/ERP, in the hope that they can be used to monitor ASD in clinical trials (84–86). This wealth of data may guide the planning for optimal biomarker choices in the **phase 2m** setting, with mechanistic markers that reflect the function of the neurobiological system(s) targeted by the treatment and other neurobehavioral outcomes that serve as more general indices of ASD symptomatology. Importantly, we will not know which markers will be the best to predict and track response until after the **phase 2m** is completed. The information yielded by this, though, would likely help contribute to improved outcomes for precision medicine optimization in phase 3—and will result in fewer trials that fail to achieve statistical significance despite having a subset of good responders. Furthermore,

intervention with an individualized approach at earlier ages is likely to have a larger effect on developmental trajectories. In combination with impactful behavioral therapies (87–91), this approach, implemented early in development, may have an even greater impact on the overall burden of ASD over a lifetime (2, 92).

Author contributions

DB generated the first draft and every other author made major contributions and agreed on the final text. All authors contributed to the article and approved the submitted version.

Conflict of interest

DB consultant for YAMO Pharma, Impel Pharma, Scioto Biosci, Quadrant Biosci, Stalicla Biosci, and Human Health. EA received consultation fees from F. Hoffmann-La Roche AG Pharmaceuticals, Impel neuropharma, Cell-El Therapeutics Ltd., ONO Pharma USA, Inc.; research funding from Roche, Anavex and Sanofi-Aventis, and SynapDx; in kind supports from AMO pharma and CRA; editorial honoraria from Wiley; book royalties from Springer and APPI, and has served on advisory boards at Roche and Quadrant Biosciences. AH consultant for IAMA Therapeutics, Jazz Pharmaceuticals, and Beaming Health. Has equity options in Quadrant Biosciences and has an investor stake in SCAN-R LLC. PW is an employee of Clinical Research Associates, LLC. CE consultant to Scioto Bioscience, Forge, and Stalicla. Nothing else to report. None of my consulting is related directly to the content of this Frontiers manuscript. TF has received funding or research support from, acted as a consultant to, received travel support from, and/or received a speaker's honorarium from the PTEN Research Foundation, SYNGAP Research Fund, Malan Syndrome Foundation, ADNP Kids Research Foundation, Quadrant Biosciences, Autism Speaks, Impel NeuroPharma, F. Hoffmann-La Roche AG Pharmaceuticals, the Cole Family Research Fund, Simons Foundation, Ingalls Foundation, Forest Laboratories, Ecoeos, IntegraGen, Kugona LLC, Shire Development, Bristol-Myers Squibb, Roche Pharma, MaraBio, National Institutes of Health, and the Brain and Behavior Research Foundation, has equity options in Quadrant Biosciences and MaraBio, and has an investor stake in Autism EYES LLC and iSCAN-R. JV-V received research funding from NIH, Simons Foundation, Roche, Janssen, Novartis, Seaside Therapeutics, Acadia, Yamo, MapLight, and SynapDx. Advisory board or consulting with Simons Foundation, Autism Speaks, Brain Behavior Research Foundation, Roche, and Novartis. Editorial stipend from Wiley.

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ORIGINAL RESEARCH
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Identifying Subgroups of Patients With Autism by Gene Expression Profiles Using Machine Learning Algorithms

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Objectives: The identification of subgroups of autism spectrum disorder (ASD) may partially remedy the problems of clinical heterogeneity to facilitate the improvement of clinical management. The current study aims to use machine learning algorithms to analyze microarray data to identify clusters with relatively homogeneous clinical features.

Methods: The whole-genome gene expression microarray data were used to predict communication quotient (SCQ) scores against all probes to select differential expression regions (DERs). Gene set enrichment analysis was performed for DERs with a fold-change >2 to identify hub pathways that play a role in the severity of social communication deficits inherent to ASD. We then used two machine learning methods, random forest classification (RF) and support vector machine (SVM), to identify two clusters using DERs. Finally, we evaluated how accurately the clusters predicted language impairment.

Results: A total of 191 DERs were initially identified, and 54 of them with a fold-change >2 were selected for the pathway analysis. Cholesterol biosynthesis and metabolisms pathways appear to act as hubs that connect other trait-associated pathways to influence the severity of social communication deficits inherent to ASD. Both RF and SVM algorithms can yield a classification accuracy level >90% when all 191 DERs were analyzed. The ASD subtypes defined by the presence of language impairment, a strong indicator for prognosis, can be predicted by transcriptomic profiles associated with social communication deficits and cholesterol biosynthesis and metabolism.

Conclusion: The results suggest that both RF and SVM are acceptable options for machine learning algorithms to identify AD subgroups characterized by clinical homogeneity related to prognosis.

Keywords: autism spectrum disorder, genomics, social cognition, language, machine learning

INTRODUCTION

Clinical heterogeneity is a norm rather than an exception in autism spectrum disorder (ASD), a complex neurodevelopmental disorder characterized by social communication deficits and stereotyped behaviors. Heterogeneous clinical features pose great challenges for diagnostics for ASD, such that children who receive a diagnosis of ASD have a range of vastly different presentations, trajectories, and outcomes. Further, the diagnostic criteria for ASD have been continuously revised through different editions of the Diagnostic and Statistical Manual for Mental Disorders (DSM), particularly the substantial changes in the 5th edition (DSM 5) where the wide range of clinical presentations have been brought together under a single ASD diagnostic entity (1). The current diagnostic system lacks an evidence-based approach and we urgently require a scientific approach to understanding which interventions are likely to be the most effective for which child with ASD (2). Accumulating evidence has shown that no pharmaceutical treatments have thus far been conclusively found to substantially reduce core symptoms of ASD (3). This may be partially attributable to the fact that most clinical trials did not take clinical heterogeneity into account and hence treatment effects remain equivocal. Variable clinical presentations may reflect different biological pathways. The identification of biomarkers for etiological pathways may hence hold the key to unraveling mechanisms underlying the variation in clinical presentations (4), which in turn may pave the way for personalized medicine in ASD.

The goal of identifying biomarkers for clinical homogeneity is to tackle challenges arising from clinical heterogeneity for research on either etiologies or treatments of ASD. One of the most extensively studied biomarkers for ASD is genetic factors. There are two different strategies to evaluate genetic markers for clinical heterogeneity: bottom-up and top-down approaches. The bottom-up approach is to define a priori subgroups using phenotypic information under the premise that some genetic loci are more likely to contribute to susceptibility to disease in a certain subgroup(s). Therefore, stratifying the population by a clinical marker (e.g., age of onset) will allow investigators to detect genetic association effects that are larger in certain subgroups. The top-down approach, on the other hand, is based on the premise that certain genetic markers can be used to distinguish subgroups, each of which is characterized by relatively homogeneous phenotypic profiles underscored by similar biological pathways—which imply similar therapeutic targets. Many of the earlier genome-wide linkage or association studies that aimed to unravel genetic underpinnings of clinical heterogeneity chose the second approach, which is to identify genetic markers associated with the phenotype defined by strict diagnostic criteria of ASD (5–7). Using the data from the Autism Diagnostic Interview-Revised (ADI-R) (8), Autism Diagnostic Observation Schedule (ADOS) (9), Vineland Adaptive Behavior Scales (VABS) (10), head circumferences, and ages at assessment as classifying variables, Veatch et al. identified clinically similar subgroups of individuals with ASD and found that the genotypes were more similar within subgroups compared to the whole population—the proportion of the total genetic variance contained in a subpopulation was 0.17 (11). However, this approach has not yielded highly replicable and clinically meaningful findings that can lead to conclusively validated etiological factors yet (12). Furthermore, another genome-wide association study of 2,576 families with ASD probands did not discover any genetic loci that exert a larger effect on the disease risk in subpopulations defined by the diagnosis, IQ, and symptom profiles; heritability estimates were also found to be similar in subpopulations to the whole population (13). Results from different groups show that an increased number of gene-truncating variants (highly pathogenic variants) may exert a considerable impact on IQ in ASD patients (14, 15); and higher burden of this pool of variants in ASD patients correlates with lower IQ scores. These studies showed that genomic approaches are able to identify genetic loci exerting larger effect on disease risk or associated with clinical outcomes, although genetic loci must be considered in an additive manner.

The top-down approach often starts with a few selected genetic loci associated with the disease. Despite fruitful findings from genome-wide and candidate gene-based association studies, few genetic loci can be used to improve accuracy in diagnostics or optimize treatment effects of therapeutics for ASD. Nevertheless, several genetic markers are found to be useful for classifying patients with ASD into relatively homogeneous subgroups. For example, Bruining et al. reported prominently higher symptom homogeneity in both the ASD group with 22q11 deletions and ASD group with Klinefelter Syndrome (KS), compared to the heterogeneous ASD sample (16). Transcriptomic profiles have also been used to identify genetic markers to classify individuals with ASD. Hu and Lai used the gene expression data to identify a subset of the "classifier" genes, which resulted in an overall class prediction accuracy of nearly 82%, ∼90% sensitivity, and 75% specificity (17). These results seem to demonstrate the value of the top-down approach.

Determining subgroups of ASD is challenging mainly because of the complexity of biological factors and clinical heterogeneity inherent to ASD. To tackle these challenges, one of the solutions is to implement state-of-the-art statistical methods that can efficiently parse through high-dimensionality data, such as machine learning (ML) algorithms, to differentiate subgroups with meaningful etiological, diagnostic, or therapeutic implications (18). Previous evidence suggests that ML algorithms can be used to reduce the number of items from standardized ASD assessment tools to make the assessment more efficient (19) and predict clinical outcomes with ASD phenotypic clusters and genetic data of copy number variations (20). The ML algorithms appear to be useful to identify phenotypic clusters as ASD subgroups that can predict clinical outcomes (21). In the current study, we attempted to implement the ML algorithms in the context of the bottom-up approach, which is to identify clusters using genomic information, and then explore the relationship between the genomic clusters and clinical features of ASD.

METHODS

Data Collection

The goal of the current study is to evaluate whether transcriptomic profiles correlated with clinical severity levels of ASD—which were measured with social communication questionnaire (SCQ) (22), can classify patients into two subgroups defined on the basis of language (i.e., the subgroup with language impairment vs. the subgroup without language impairment). The language function is considered as a strong predictor for cognitive ability and adaptive skills in children with ASD (23), and its variation within ASD patients is influenced by genetic factors (24–26). The presence of language impairment was defined as the total score (verbal) >10 in the section of Qualitative Abnormalities in Communication in Autism Diagnostic Interview-Revised (ADI-R) (27). A total of 31 children diagnosed with ASD were recruited in the current study. The clinical diagnoses were made by Gau, a board-certified child psychiatrist, and confirmed by the ADI-R interview with the parents. The Chinese version of the ADI-R been approved by the Western Psychological Services in May 2007 (28) mRNA was extracted from lymphoblastoid cell lines (LCL) of all participants. The microarray experiment was performed at the Core Laboratory of National Taiwan University Hospital in Taiwan, using the Affymetrix Human Genome U133 Plus 2.0 Array (Affymetrix Inc., Santa Clara, CA, USA). The experimental procedures followed the protocols provided by the manufacturer. The study was conducted with the ethical approval by the Institutional Research Board at National Taiwan University Hospital in Taiwan.

Statistical Methods

Transcriptome-Wide Association Analysis

We evaluated the integrity of 28S and 18S rRNA by electrophoresis of 2 mg of total RNA in 1.2% agarose gel containing 2.2 M formaldehyde and in a running buffer containing 0.2 M of MOPS (pH 7.0), 20 mM of sodium acetate and 10 mM of EDTA (pH 8.0). The A260/A280 ratio was used to measure the quality of RNA. The ratio between 1.9 and 2.1 was considered good quality. The intensity files of all the subjects were input into the computer program GAP: Generalized Association Plots (29, 30) for quality control using visualization and descriptive statistics. We used the Robust Multi-array Analysis (RMA) method to normalize the data (31). In order to filter out probe sets with low variations and to reduce the impact of multiple comparisons, we kept only the 1,000 probe sets with the largest standard deviations. We searched for differential expression regions (DERs) by prioritizing the gene expression levels associated with the clinical severity indicated by SCQ scores, we used the generalized linear model to screen for probes across the whole genome with mRNA levels associated with the SCQ scores with unadjusted $p < 0.00001$. All original intensity ratio data were transformed into logarithmic 2 values after being normalized. We controlled for the batch effect by adjusting for the batch as a binary covariate since there were two batches. These probes constitute the primary source of predictors to determine ASD subgroups.

Gene Ontology and Pathway Analysis

The DERs with a fold-change >2 were selected for the gene ontology and pathway analysis to evaluate the biological relevance and functional pathways of the significant genes. We have incorporated the KEGG (32), WikiPathways (33), BioCarta (34), and Reactome (35) pathway database for the cell signaling pathways. We have also considered the GO Biological Process (2018) database for gene ontological analysis (36). The GO terms and pathways enriched by the list of genes were identified using the hypergeometric analyses with an adjusted $P \leq 0.05$ was considered as statistically significant.

Gene Over-Representation Analysis

Then we used the webtool at ConsensusPathDB (http://cpdb.molgen.mpg.de/) to identify pathway-pathway interaction network (CPDB analysis) (37). The analysis criteria included: (1) one-next neighbors for the radius with $p < 0.01$, (2) pathway-based sets at least two overlapped genes and $p < 0.01$, and (3) gene ontology level 2 categories with $p < 0.01$. The results from the second approach helped visualize the possible "hub" pathway from the top 10 networks associated with the candidate genes.

We chose two machine learning (ML) algorithms to evaluate the clustering results: random forest classification and support vector machine algorithms. The presence of language impairment was considered as a dichotomous clinical outcome to determine classification errors. We chose the first ML algorithm proposed by Shi and Horvath (38). We used the Random Forest classification (RF) algorithm in an unsupervised mode to generate a proximity matrix. The gene expression data were analyzed using RF using two different approaches for comparison. The first approach is to reduce data dimensionality using principal component analysis to identify principal component (PC) scores for each subject. The top 10 PCs were selected to calculate the proximity matrix that provides a rough estimate of the distance between samples based on the proportion of times the samples end up in the same leaf node. The proximity matrix values were then converted to a dissimilarity matrix to classify the sample into two subgroups using partitioning around medoid (PAM) (39). The second approach is to use the information of all 191 probes with gene expression levels significantly associated with SCQ scores to generate the RF proximity matrix. Similarly, the RF proximity matrix was used to classify the sample into two subgroups using the PAM clustering analysis (39) to classify the patients into two clusters to determine the final cluster assignment. The RF-PAM clustering analysis could allow us to evaluate the classification error by calculating the frequency of patients with language impairment in the cluster, in which the majority of patients had no language impairment, and vice versa.

We further chose Support Vector Machine (SVM) as the second ML algorithm to classify the patients into two subgroups (40). To reduce data dimensionality, we implemented principal component analysis to identify the principal component (PC) scores for each subject. The data of PC scores were split in a 7:3 ratio—in other words, 70% of the data was used for training the model and the remaining 30% was for testing the model. Estimating the C (Cost) parameter to classify

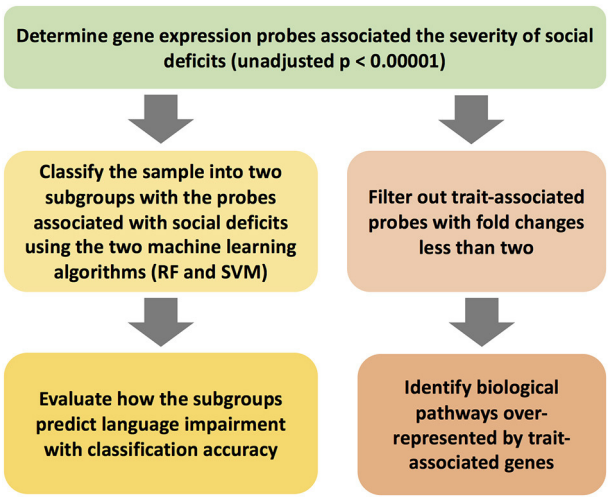

FIGURE 1 | The workflow of the study scheme.

TABLE 1 | Clinical features of the patients in the current study.

	Language impairment (51.3%)	No language impairment (48.7%)	Relationship with language impairment*
Age	9.00 (*SD*: 2.52)	8.91 (*SD*: 3.99)	$P > 0.05$
ADIR-BV	17.83 (*SD*: 3.27)	8.55 (*SD*: 1.13)	$P < 0.0001$
ADIR-BN	8.92 (*SD*: 2.71)	3.64 (*SD*: 1.43)	$P < 0.0001$
SCQ	22.19 (*SD*: 4.84)	11.47 (*SD*: 4.84)	$P < 0.0001$
VIQ	82.08 (*SD*: 20.77)	111.91 (*SD*: 10.12)	$P = 0.0003$
PIQ	90.83 (*SD*: 15.74)	101.36 (*SD*: 15.34)	$P > 0.05$
SRS	89.61 (*SD*: 16.12)	79.55 (*SD*: 27.99)	$P > 0.05$

ADIR-BV, Autism Diagnostic Interview–Revised, Qualitative Abnormalities in Communication, Total Verbal score. ADIR-BN, Autism Diagnostic Interview–Revised, Qualitative Abnormalities in Communication, Total Non-Verbal score. SCQ, Social Communication Questionnaire score; VIQ, verbal IQ; PIQ, performance IQ; SRS, Social Responsiveness Scale score.

**The student's t-test was performed to evaluate whether the the two subgroups classified by the presence of language impairment had different values in each continuous variable.*

the data was performed using SVM with the linear kernel function. The choice of kernel function was made based on the recommendation from a prior study that using microarray data to predict the diagnosis of colon cancer—which concludes that linear kernel function leads to a lower prediction error than the RBF, quadratic, and polynomial kernel functions (41). The prediction accuracy and Kappa value estimated when the C value was held constant at 1. The Kappa value was calculated using the formula $(p_o - p_e)/(1\text{-}p_e)$, where p_o and p_e denote the observed agreement and expected agreement for classification, respectively. We further used the confusion matrix, which contains the number of correct and incorrect predictions summarized with count values and broken down by each class, to predict the prediction accuracy of the SVM model. The accuracy is calculated as $(TP + TN)/(TP+TN+FP+FN)$, where TP and TN refer to true positives and true negatives, respectively; FP and FN refer to false positives and false negatives, respectively. These two measures (i.e., accuracy and Kappa value) were chose to evaluate the SVM performance as recommended by previous studies (42, 43). The Kappa statistics could lead to a biased performance estimate in unbalanced situations (44), which is not the characteristic of the current sample. The SVM analysis was performed using the R package "*caret*" (45).

RESULTS

The workflow of the current project is shown in **Figure 1**. The clinical features of the 31 subjects are summarized in **Table 1**. The group with language impairment and the group without language impairment has significant differences in clinical features associated with both social communication function and verbal IQ scores.

The transcriptomic association study reveals 191 probes that were statistically significantly associated with SCQ scores with a $p < 0.00001$. The batch effect seemingly did not affect the association results (**Supplementary Figure 1**). We selected 54 of them with a fold-change >2 for the pathway analysis. Differentially expressed 54 genes with logarithmic fold changes

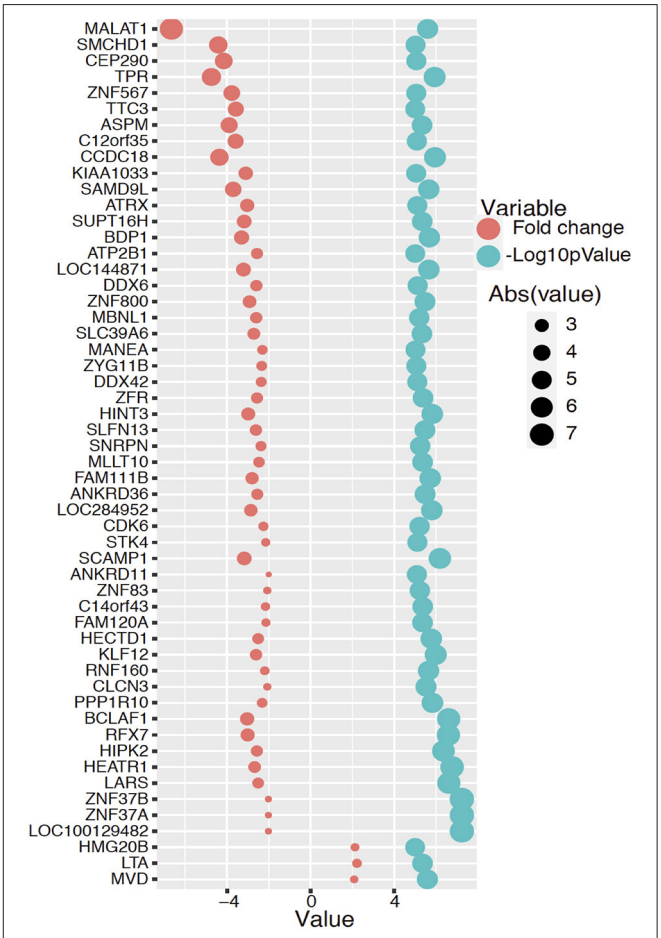

FIGURE 2 | Differentially expressed 54 genes with fold changes and –logarithmic 10 adjusted *p*-values. The red circle represents logarithmic fold change and the blue color circle represents –logarithmic 10 adjusted *p*-value for each significant gene.

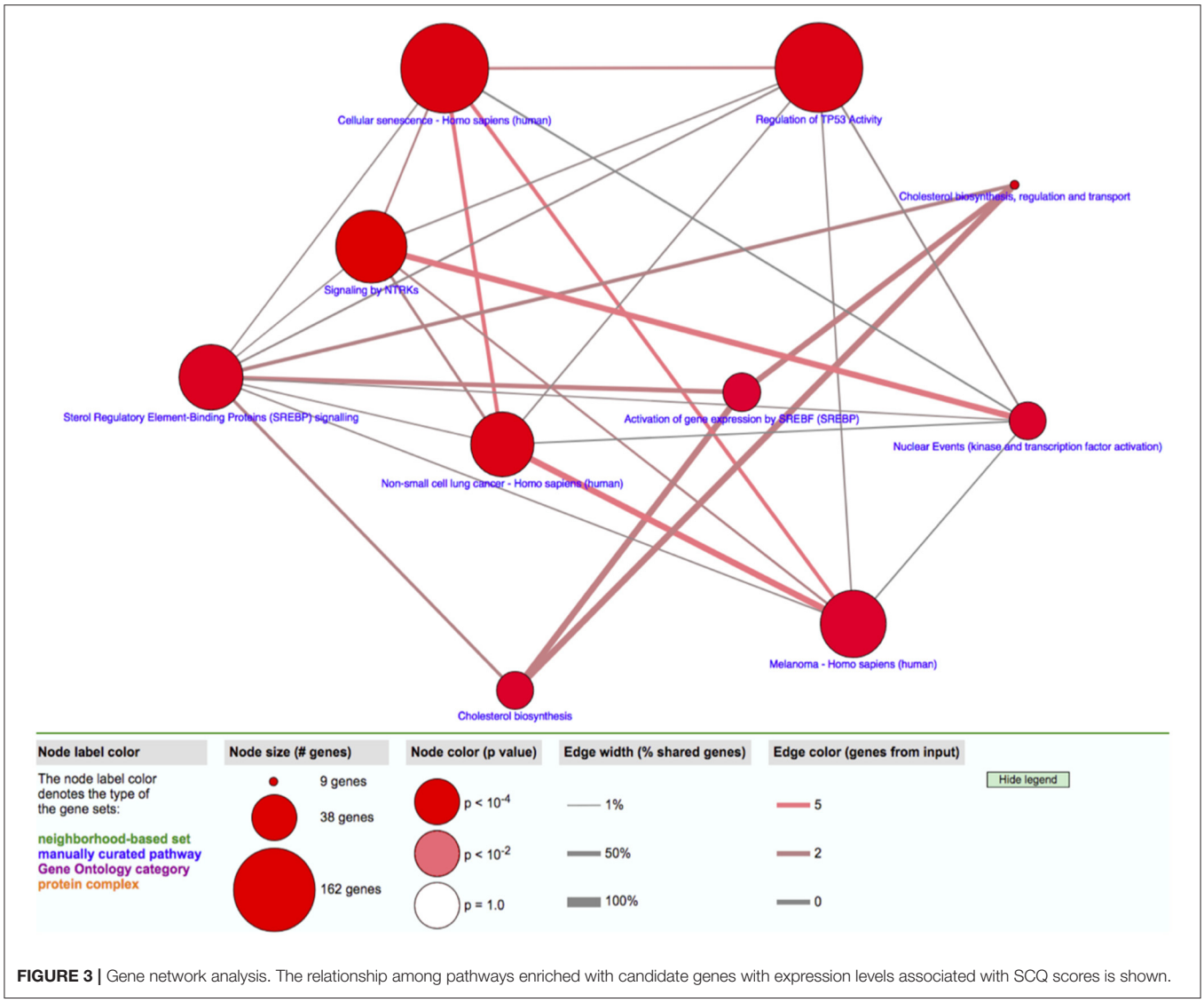

FIGURE 3 | Gene network analysis. The relationship among pathways enriched with candidate genes with expression levels associated with SCQ scores is shown.

and –logarithmic 10 adjusted *p*-values are listed in **Figure 2**. Only three pathways were found to be over-represented by these 54 genes with adjusted $p < 0.05$: cholesterol biosynthetic process (GO:0006695), secondary alcohol biosynthetic process (GO:1902653), and regulation of signal transduction by p53 class mediator (GO:1901796). The CPBD analysis shows that Sterol Regulatory Element-Binding Proteins (SREBP) signaling pathway is the pathway connected with 9 of the 10 pathways including cholesterol biosynthetic pathway, so it can be regarded as the "hub" associated with genetic network for ASD (**Figure 3**). This pathway of SREBP focuses on the regulation of lipid metabolism by SREBP.

The RF-PAM analysis identified two clusters (**Figure 4**). The classification accuracy was 67.7% when the top 10 PCs were used to generate the proximity matrix, while the classification accuracy was 96.9% when all 191 probes were used to generate the proximity matrix. The SVM analysis based on the top 10 PC scores shows that the clustering results reached classification accuracy at 93.3% (95% CI 68.1–99.8%) and no-information rate (i.e., the largest proportion of the observed classes) at 53.3% ($p = 0.0011$). Other parameters relevant to prediction performance include Kappa value = 0.86, sensitivity = 0.86, specificity = 1.00, and balanced accuracy = 0.93. The SVM analysis using the information of all probes with differential gene expressions associated with SCQ scores yielded a slightly higher classification accuracy than the SVM analysis based on the top 10 PC scores. The classification accuracy at 99.9% (95% CI 78.2–100%) and no-information rate (i.e., the largest proportion of the observed classes) at 53.3% ($p = 8.035 \times 10^{-5}$) were achieved when 191 probes were analyzed. This classification accuracy can be demonstrated in gene expression level distributions stratified by language impairment (**Supplementary Figure 2**). The SVM clustering results are shown in **Figure 5**. The results suggest that the first two principal components could identify support vectors that fell in the area with better prediction confidence (**Figure 5A**), compared with the results predicted by individual

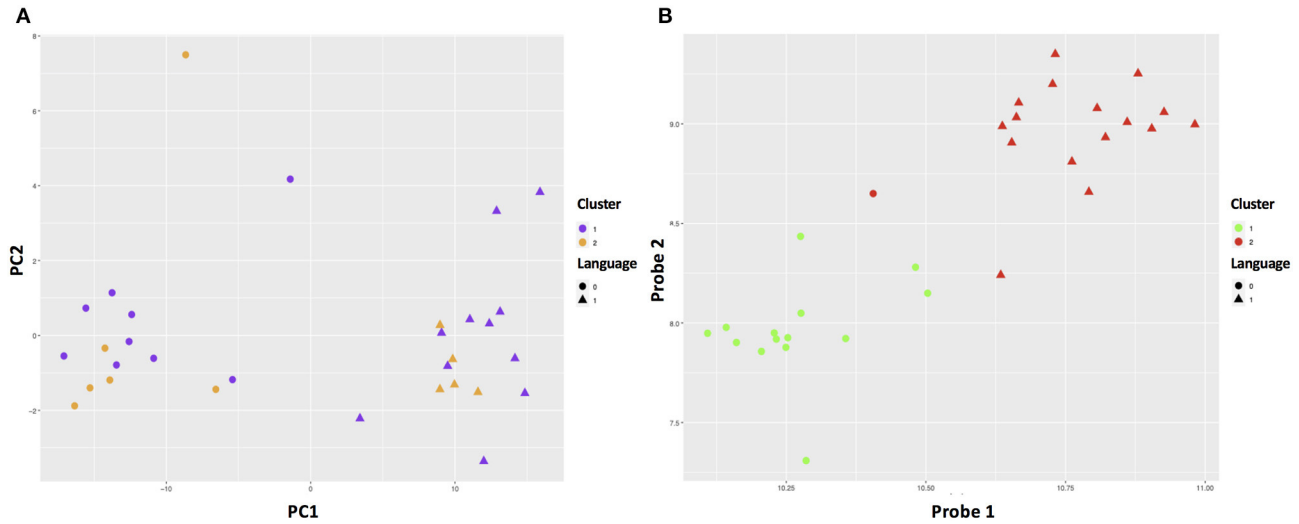

FIGURE 4 | ASD subgroups identified using RF and PAM clustering algorithms. Dim1 and Dim2 correspond to principal components 1 and 2, respectively. **(A,B)** The results based on the top 10 principal components (PCs) and the 191 probes, respectively. We used the first two predictors to make the plots to demonstrate how different approaches classified the sample.

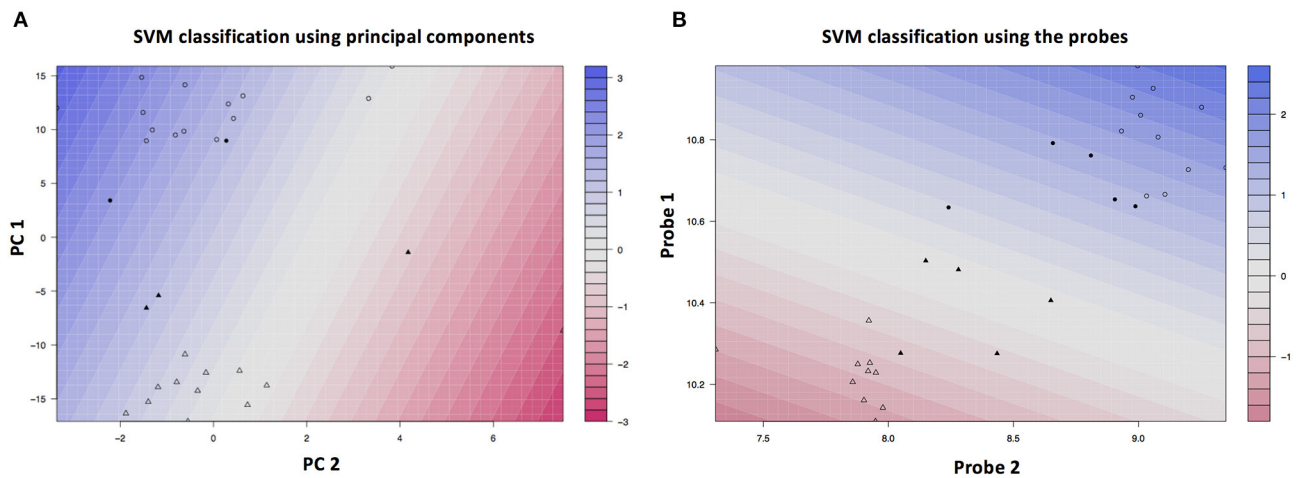

FIGURE 5 | SVM clustering results based on the top PCs. **(A)** Shows the color gradient that indicates how confidently a new point would be classified based on its features. PC1 and PC2 represent the first and second principal components, respectively. **(B)** Shows the color gradient that indicates how confidently a new point would be classified based on its features when predictors were based on all SCQ-associated probes. Probe 1 and probe 2 represent the first and second probes, respectively. The solid symbols indicate the support vectors and the hollow circles indicates other subjects. The circles and triangles represent the first and second subgroups, respectively.

probes (**Figure 5B**). The predicting performance of the RF-PAM and SVM algorithms is listed in **Table 2**.

DISCUSSIONS

We conducted a proof-of-concept study to demonstrate how transcriptomic data from a small sample could provide useful biomarkers to classify ASD subgroups. The selection of the predictors was based on DERs associated with SCQ scores, which indicate the variation in severity levels of social communication deficits, a hallmark clinical feature of ASD. The DER with strongest evidence for the association with social deficits in our sample is matched with the HEATR1 gene (HEAT Repeat Containing 1). The HEART1 gene is associated with schizophrenia (46). The HEATR1 gene abnormalities in the brain during the embryonic stage has been reported in zebrafish (47). The candidate genes that harbor these DERs suggest several genetic pathways that modulate the variation in social communication functions. Among these pathways, the pathway

TABLE 2 | Predicting performance of two machine learning algorithms.

Method	Predictors	Prediction accuracy
RF-PAM	191 probes	96.90%
RF-PAM	10 PC*	67.70%
SVM	191 probes	99.90%
SVM	10 PC*	93.30%

**Principal component.*

of cholesterol biosynthesis/metabolism and sterol regulatory element-binding proteins (SREBP) pathway—cholesterol metabolism appear to act as hubs that connect other top SCQ-associated pathways. Particularly, the SREBP pathway shares most genes with other SCQ-associated pathways. These two pathways are related to lipid metabolism. Cholesterol synthesis and uptake are tightly modulated at the transcriptional level through negative feedback control, which is regulated by SREBPs (48). The relationship between lipid metabolism and brain functions has been well-documented. A growing body of evidence has indicated that cholesterol metabolism plays a key role in synaptic functions (49–51). Dysregulated cholesterol metabolism has been extensively documented in ASD (51–58). A recent study implemented a personalized medicine approach combining healthcare claims, electronic health records, familial whole-exome sequences, and neurodevelopmental gene expression patterns, and identified an ASD subtype characterized by dyslipidemia (59). There are certainly several other genetic pathways involved in molecular mechanisms underlying social communication deficits. Nevertheless, our results indicate that cholesterol synthesis/metabolism pathways act as hubs that connect most other biological pathways, which suggest that the genomic functional changes associated with lipid metabolism may moderate other genomic changes, such as the p53 signaling pathway, that regulate social communication functions.

Using the DERs as biomarkers, we clustered the sample into two subgroups using two different ML algorithms. Both the RF-PAM and SVM analyses yielded similar levels of classification accuracy when all 191 markers were utilized. However, compared to the analysis using the RF-PAM algorithm, the analysis using the SVM algorithm seemed to be more robust when we performed dimension reduction for all the 191 markers with the PCA method. The RF algorithm is applicable when there are more predictors than observations, relatively insensitive to the noise (e.g., a large number of irrelevant genes), and does not rely on excessive fine-tuning of parameters (60). RF algorithm is more robust to small sample size as the SVM algorithm (61, 62). However, Brown et al. found that SVM outperforms other techniques that include Fisher's linear discriminant, Parzen window, and tow decision tree learners when using gene expression data to predict clinical outcomes (63). Additionally, Statnikov et al. conducted a comprehensive comparison of RF and SVM using microarray data for 22 diagnostic and prognostic datasets and concluded that SVM is superior to RF in terms of classification accuracy (64). Although the purpose of this study is not to comprehensively evaluate which ML algorithm outperforms the other ML algorithm, our results seem to lend some support to the robustness of the SVM algorithm. Nevertheless, the RF algorithm is at least as robust as the SVM algorithm when the dimension of input variables is not substantially reduced.

One of the major limitation of the current study is the small sample size. Nevertheless, some machine learning algorithm, such as SVM, can handle a small sample with a large number of features. Additionally, model overfitting may arise due to a lack of another independent sample for validation. Furthermore, unknown confounders may cause spurious associations between the phenotype and genomic markers. However, the goal of this proof-of-concept study is prediction of subtypes rather than the identification of etiologies. Therefore, confounders would not yield a substantial impact on prediction results (65).

The clinical and etiological heterogeneity in ASD has meant that there is considerable variability in treatment outcomes across different interventions and between individuals receiving the same intervention. Hence the traditional diagnostic and "one size fits all" approach to ASD intervention needs improvement. Further, we currently do not have a sufficient understanding of "what would work for whom," thereby limiting opportunities for maximizing outcomes for children and their families with economic ramifications for broader society. In this context, ML algorithms have been found to be useful in predicting diagnostic accuracy in ASD with neuroimaging data (66). Further, one recent study used Gaussian Mixture Models and Hierarchical Agglomerative Clustering, which provide a statistical framework for learning latent cluster memberships to determine ASD subgroups with differentiated treatment responses (67). Our findings that using ML algorithms, children could be classified into two groups based on the presence of language impairment, offers promise for unraveling clinically meaningful subgroups in ASD. This, in turn, can be used for predicting likely responsiveness (and non-responsiveness) to specific treatment pathways. This "precision" approach to assessment and intervention will ensure that resources for appropriate intervention and supports are allocated in an evidence-based manner. This is critical as without timely recognition of the variability in the clinical presentation, neurocognitive level of functioning, and psychosocial circumstances coupled with individualized intervention, children and their families may miss key opportunities of brain plasticity available in the critical early years. ML techniques as utilized in this study offer a viable solution to address this by allowing matching interventions and supports that are tailored to the individual profile and needs.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://figshare.com/articles/dataset/Autism_gene_expression_data/14251328.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Research Ethics Committee of the National Taiwan University Hospital. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

P-IL and MM carried out the statistical analysis. P-IL and VE conceived of the study and drafted the manuscript. SG participated in the study design and coordination. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpsyt.2021.637022/full#supplementary-material

Supplementary Figure 1 | The evaluation of potential batch effect due to the microarrays timing. **(A)** The kernel density distributions of gene expression levels of the two batches are shown. **(B)** Time 1 and time 2 indicate the association test results that adjusted for the time (i.e., batch) vs. the results without adjusting for the time.

Supplementary Figure 2 | Randomly selected four probes associated with SCQ scores stratified by the presence of language impairment. The red and blue curves represent the group without language impairment and the group with language impairment, respectively.

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BRIEF RESEARCH REPORT
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Brief Report: Preliminary Evidence of the N170 as a Biomarker of Response to Treatment in Autism Spectrum Disorder

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Background: Autism Spectrum Disorder (ASD) is a neurodevelopmental disorder characterized by primary difficulties in social function. Individuals with ASD display slowed neural processing of faces, as indexed by the latency of the N170, a face-sensitive event-related potential. Currently, there are no objective biomarkers of ASD useful in clinical care or research. Efficacy of behavioral treatment is currently evaluated through subjective clinical impressions. To explore whether the N170 might have utility as an objective index of treatment response, we examined N170 before and after receipt of an empirically validated behavioral treatment in children with ASD.

Method: Electroencephalography (EEG) data were obtained on a preliminary cohort of preschool-aged children with ASD before and after a 16-week course of PRT and in a subset of participants in waitlist control (16-weeks before the start of PRT) and follow-up (16-weeks after the end of PRT). EEG was recorded while participants viewed computer-generated faces with neutral and fearful affect.

Results: Significant reductions in N170 latency to faces were observed following 16 weeks of PRT intervention. Change in N170 latency was not observed in the waitlist-control condition.

Conclusions: This exploratory study offers suggestive evidence that N170 latency may index response to behavioral treatment. Future, more rigorous, studies in larger samples are indicated to evaluate whether the N170 may be useful as a biomarker of treatment response.

Keywords: autism spectrum disoder, electroencephalography, N170, biomarker, pivotal response treatment

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INTRODUCTION

Autism spectrum disorder (ASD) is a heterogeneous neurodevelopmental disorder hallmarked by difficulties with social communication, along with restricted and repetitive behaviors and atypical response to sensory information (1). Without objective biomarkers for ASD, clinical practice and research are reliant on subjective clinician judgments. There is a critical need to identify objective biomarkers for ASD to enhance clinical research by providing quantifiable indices of functional processes relevant to ASD, such as face perception.

The N170 is a well-studied neural marker of face perception. This face-sensitive event-related potential (ERP) is evident as a negative deflection over occipitotemporal scalp ∼170 milliseconds (ms) after viewing a face and indexes structural encoding, an early stage of face processing (2). The latency of the N170 reflects temporal processing of faces, such that longer latencies reflect slower, less efficient face processing or incomplete developmental maturation. Delayed N170 latency is observed in individuals with ASD relative to age- and IQ-matched typically developing (TD) children (3), a neuroscientific finding that is reproducible across heterogeneous ASD samples (4). Right hemisphere N170 latency to upright faces was recently accepted into the FDA Center for Drug Evaluation and Research (CDER) Biomarker Qualification Program, making it the first biomarker for a psychiatric disease to receive this designation (5).

Most N170 studies to date have focused on establishing group mean differences and relationships with symptomatology. These features are germane to multiple biomarker contexts of use, such as stratification into treatment-relevant subgroups; however, they provide limited information regarding its potential utility in other desired contexts of use, such as quantifying change in neural systems in response to treatment. Determination of viability in this context of use requires appropriately designed studies that measure N170 latency in children with ASD in the context of intervention and associated change in clinical status.

Very few studies have examined N170 latency as a potential index of treatment response. Dawson et al. (6) examined neural correlates of face perception subsequent to early behavioral intervention in 48- to 77-month-old children. Though differences in the Nc, an attention-related ERP arising from the prefrontal and anterior cingulate cortices (7), in response to faces was observed between intensive intervention and community treatment groups, significant differences were not observed at the N170. This study indicates the appropriateness of face processing circuitry for quantifying response to treatment. It is, however, difficult to draw conclusions regarding the appropriateness of the N170 as a biomarker of treatment response from these data given the absence of pre-intervention EEG recordings and the consequent inability to conduct within-participant comparisons of N170 change in response to treatment. A second study examined changes in N170 in response to a drama-based social skills group intervention and failed to detect changes associated with treatment relative to a waitlist control (8). Given evidence that social skills groups, administered at a lower intensity level than individualized behavioral treatments, do not consistently improve face perception [(9); but see (10)], it is possible that the systems indexed by the N170 were not affected by this treatment. Conclusions regarding the potential utility of the N170 in this study are also complicated by the observed improvements on measures of face perception and shorter N170 latencies at posttest relative to baseline, suggesting placebo effects could have obscured associations with change in N170.

The current study sought to explore the potential utility of N170 latency as an index of treatment response in a preliminary study designed to address several of the limitations of prior research. Like previous studies, we examined children over the course of receipt of an empirically validated intensive and individualized intervention, in this case, pivotal response treatment [PRT; (11)]. To build on prior research and potentially improve sensitivity to evaluate change in a neural biomarker, we: (1) collected pre- and post-test data to permit intra-individual comparisons; (2) administered treatment individually rather than in a group setting and focused specifically on social-communication; (3) administered treatment over an extended period of time (16 weeks) and with a high level of intensity to increase the likelihood of changing neural systems; (4) utilized a treatment already demonstrated to enact change in social perceptual brain systems (12).

We hypothesized that children would exhibit behavioral improvement in response to PRT and that right hemisphere N170 latency, commonly increased in ASD relative to TD children, would decrease in response to treatment. In contrast, we predicted that the P100, a positive-going component arising from the parieto-occipital region ∼100 ms after stimulus presentation and reflecting low-level visual processing (13, 14), would not be affected by social-communicative treatment.

METHODS

Participants

Seven 4- to 7-year-old children with ASD received a 16-week course of PRT as part of an ongoing research study at the Yale School of Medicine (**Table 1**). Of these seven participants, three served in a waitlist control condition prior to enrolling in treatment, and five served in a follow-up condition conducted 16 weeks after the end of PRT. All study participants met gold-standard diagnostic criteria for ASD according to the Autism Diagnostic Observation Schedule [ADOS; (15)] and the Autism Diagnostic Interview-Revised [ADI-R; (16)] and had IQs > 70. PRT targeted social communication skills and play for 8 hours per week, which involved direct work with the child and parent in clinic and at home.

EEG Recording Procedure

EEG data collection was attempted at four time points: 16 weeks prior to the start of treatment (for the waitlist control group only), pre-treatment, post-treatment, and 16 weeks after the conclusion of treatment (follow-up). Participants were included in analysis if they contributed good quality data for both pre- and post-treatment visits. Thus, fewer participants have data for the waitlist control and follow-up EEG sessions.

The EEG paradigm consisted of 70 computer-generated, grayscale faces (35 male and 35 female) displaying neutral and fearful affect. Participants viewed 146 dynamic trials in random sequence, lasting a total of 15 minutes. There were 70 neutral to fearful faces, 70 fearful to neutral faces, and a total of 6 targets to maintain attention (17).

Each trial consisted of a central fixation crosshair presented for 200–300 ms followed by a static face with either a neutral or fearful expression appearing on the center of the screen for 500 ms. Afterward, the face changed from either neutral to fearful or fearful to neutral expression in an animated, realistic movement. This second face was also presented on the center

TABLE 1 | Participant information at the pre-PRT timepoint.

Participant	Age (years)	DAS	ADOS total	SRS T-score	VABS socialization domain standard score
1	5.78	127	12	68	95
2	7.01	95	26	70	80
3	5.16	121	24	68	88
4	4.51	122	11	61	88
5	6.35	106	19	61	97
6	5.48	110	11	78	81
7	4.59	105	22	72	85
Mean	5.55	112.3	17.9	68.3	87.7

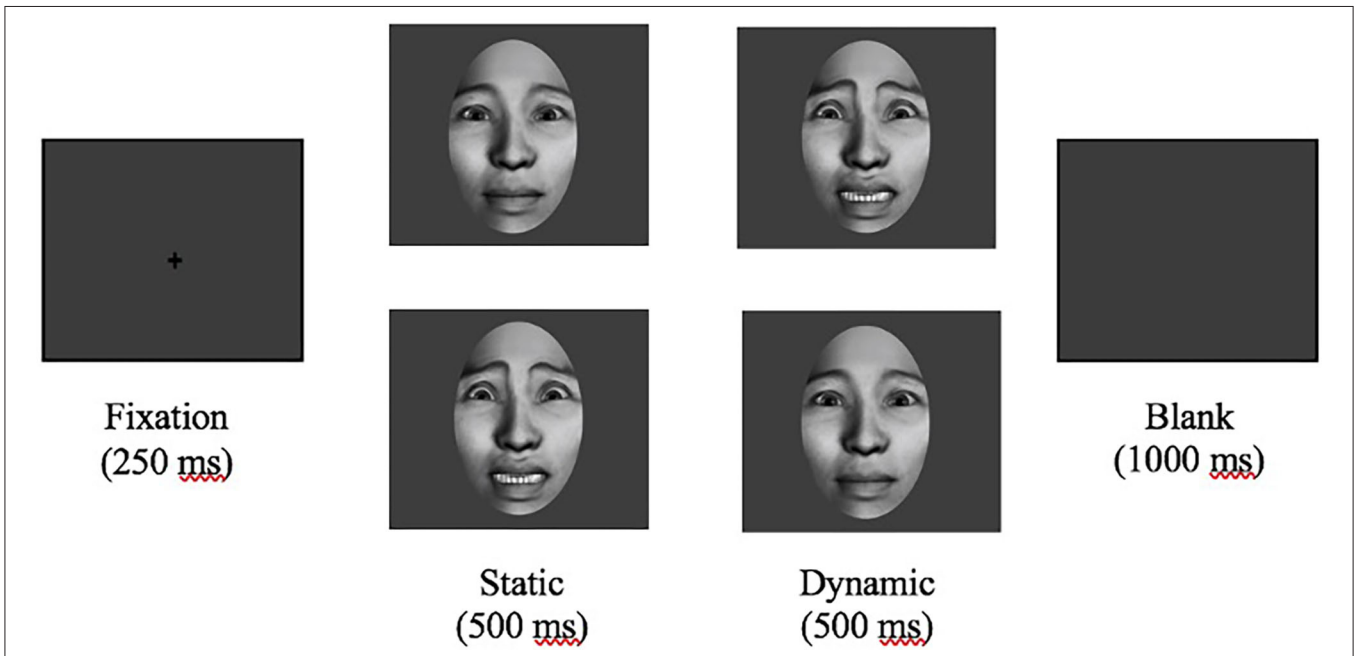

FIGURE 1 | Experimental paradigm. After a 200–300 ms fixation crosshair, a static neutral or fearful face was presented for 500 ms before dynamically changing expression. ERP data was segmented to an epoch 100 ms before to 500 ms after presentation of the initial static face.

of the screen for 500 ms (**Figure 1**, **Supplementary Material 1**). In total, faces were presented for 1,000 ms for each trial. During the paradigm, participants were instructed to press a button in response to a target stimulus, white balls, interspersed throughout the paradigm to maintain attention. A behavioral assistant was seated with all participants throughout EEG recording to monitor attention and limit participant movement.

EEG Analysis

EEG was recorded with a 128-channel Geodesic sensor net. Data was analyzed offline using NetStation 4.5.4. Data was filtered at 0.1–30 Hz and then segmented to an epoch 100 ms before to 500 ms after presentation of the initial static face. Data was baseline corrected to the 100 ms preceding stimulus presentation and re-referenced to an average. Trials with eye movements and blinks were detected and excluded using NetStation's eyeblink and eye-movement algorithms (±100 μV threshold for eye movements and ±140 μV for eye blinks). Channels were marked bad in each trial if they exceeded 200 μV for the entire trial and based on visual inspection. If channels were marked bad in more than 40% of trials, the channel was marked bad for all remaining trials. If a trial contained more than 10 bad channels (>15%), eye blinks, or other eye movements, it was excluded from further analysis. If a trial contained fewer than 10 bad channels, the bad channels were replaced using spherical spline interpolation (18). Trial by trial data were averaged at each electrode for the fear and neutral conditions for each participant. Participants were required to have 15 good trials per condition to be included for analyses, so all included participants had at least 30 adequate trials. ERP data were averaged over the right occipitotemporal region [(19); electrodes 89, 90, 94, 95; **Figure 2A**], consistent with previous research showing that neural regions specialized for face perception, namely the fusiform face area and superior temporal sulcus, are lateralized to the right hemisphere (20). Temporal windows for the P100 and N170 were 88–160 ms and 180–282 ms, respectively, based on maximal amplitude in grand

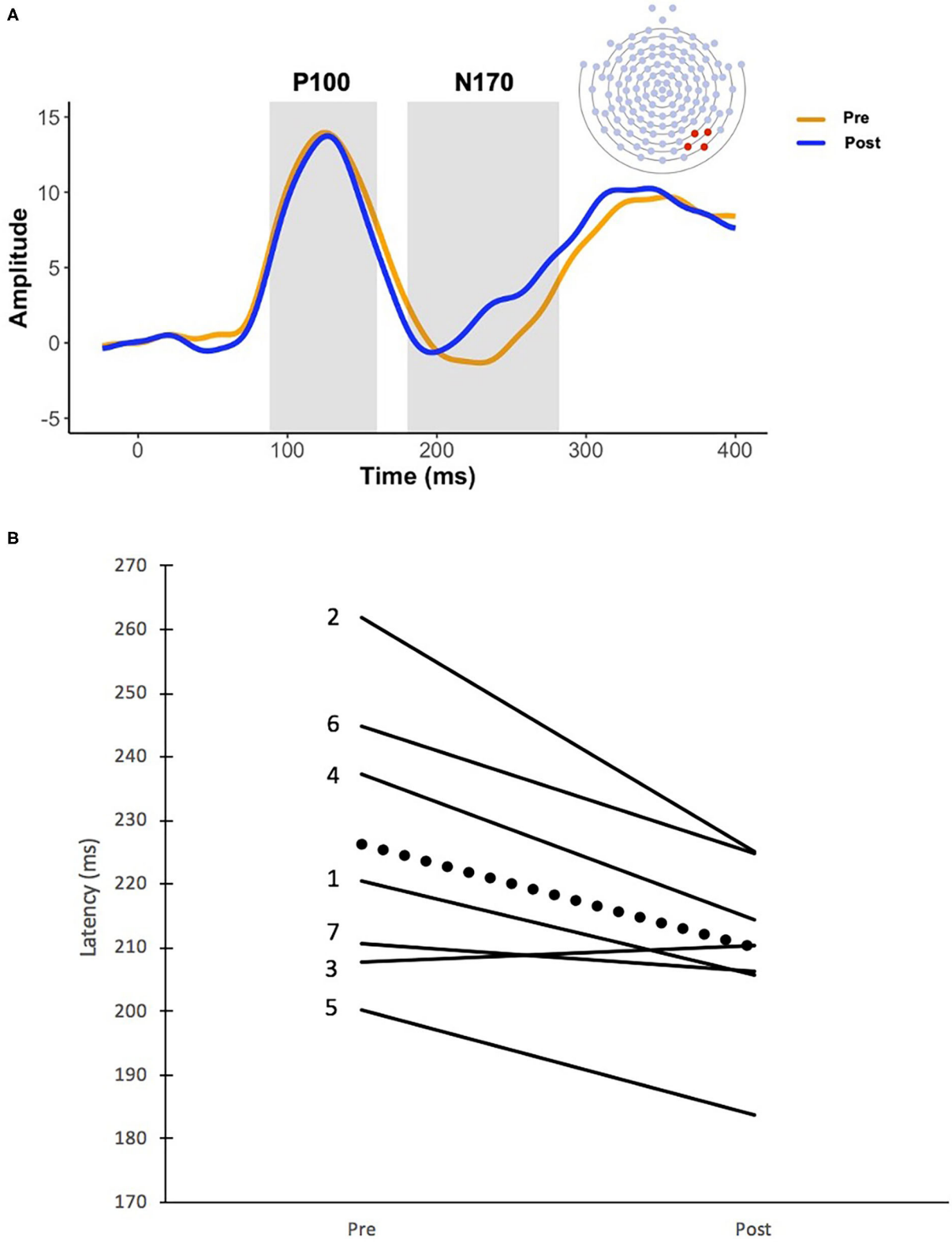

FIGURE 2 | (A) Grand averaged waveforms before and after 16 weeks of PRT. The orange line represents the recording prior to PRT and the blue line represents the recording after PRT. Temporal windows for the P100 (88–160 ms) and the N170 (180–282 ms) are represented by the gray boxes, and the P100 and N170 components are labeled accordingly. N170 electrode recording sites are also depicted. ERP data were averaged over the right occipitotemporal region (electrodes 89, 90, 94, 95). **(B)** Changes in N170 latency before and after 16 weeks of PRT. Solid lines indicate individual change in N170 latency for each of the seven subjects and the dotted line represents average group change. Numbers to the left of each line correspond with participant numbers listed in **Table 1**.

averages and confirmed in individual averages. Latency and amplitude of the maximal peak within the 88–160 ms window were extracted for the P100. Latency and amplitude of the minimal peak within the 180–282 ms window were extracted for the N170.

Statistical Analysis

P100 amplitude, P100 latency, N170 amplitude, and N170 latency were analyzed using four separate repeated measures analysis of variance (ANOVA) with pre-treatment and post-treatment ERP components as within-subject factors. N170 latencies to neutral and fearful faces were comparable pre- and post-PRT ($p = 0.97$, $p = 0.35$), as were N170 amplitudes ($p = 0.70$, $p = 0.58$). Similarly, P100 latencies to neutral and fearful faces were comparable pre- and post-PRT ($p = 0.58$, $p = 0.21$), as were P100 amplitudes ($p = 0.59$, $p = 0.29$). As a result, fearful and neutral conditions were collapsed at each time point for each ERP component. When significant, follow-up repeated measures ANOVAs were used to compare pre-ERP data to the waitlist control subset and post-ERP data to the follow-up condition subset. Additionally, changes in clinical symptomatology reflected in total ADOS scores (15), overall Social Responsiveness Scale (SRS) T-scores (21), and the socialization domain of the Vineland Adaptive Behavior Scales [VABS; (22)] were analyzed using repeated measures ANOVAs. When applicable, the relationships between changes in ERP data and changes in clinical symptomatology were analyzed using Pearson's correlations.

RESULTS

Behavioral Measures

Children participating in treatment displayed significant reductions in total ADOS scores, indicating an improvement in clinical symptomatology [$F_{(1,6)} = 12.67$, $p = 0.012$, $\eta_p^2 = 0.679$]. Significant changes in SRS T-scores [$F_{(1,6)} = 1.20$, $p = 0.315$, $\eta_p^2 = 0.167$], the VABS socialization domain [$F_{(1,s6)} = 1.08$, $p = 0.340$, $\eta_p^2 = 0.152$], and socialization subdomains (ps > 0.05) were not observed.

ERP Results

P100

No significant change was observed in either P100 latency [$F_{(1,6)} = 0.88$, $p = 0.384$, $\eta_p^2 = 0.128$] or P100 amplitude [$F_{(1,6)} = 0.037$, $p = 0.854$, $\eta_p^2 = 0.006$] between pre- and post- time points.

N170

A main effect of time point indicated a significant reduction in N170 latency after PRT [$F_{(1,6)} = 11.18$, $p = 0.016$, $\eta_p^2 = 0.651$], with average N170 latency decreasing from 226 ms (SD = 22.48 ms) to 210 ms (SD = 14.05 ms) (**Figures 2A,B**). There was no significant change in N170 amplitude before and after treatment [$F_{(1,6)} = 2.70$, $p = 0.152$, $\eta_p^2 = 0.310$].

Waitlist and Follow-Up

Given statistically significant decreases in N170 latency between pre- and post-treatment, exploratory analyses compared differences with the waitlist ($n = 3$) and follow-up ($n = 5$) subgroups. There was no significant change in N170 latency in the 16-week period from the waitlist condition to the start of PRT [$F_{(1,2)} = 2.50$, $p = 0.255$, $\eta_p^2 = 0.556$] and also no change in N170 latency in the 16-week period from the end of PRT to the follow-up condition [$F_{(1,4)} = 5.48$, $p = 0.079$, $\eta_p^2 = 0.578$].

Relationship Between N170 and Behavioral Measures

No significant correlations between electrophysiological changes in N170 latency and changes in total ADOS scores ($r = -0.275$, $p = 0.551$), SRS scores ($r = -0.314$, $p = 0.493$), Vineland socialization domain scores ($r = -0.165$, $p = 0.723$), or socialization subdomain scores (ps > 0.05) were found.

DISCUSSION

Consistent with our hypotheses, significant reductions in N170 latency were observed in 4- to 7-year-old children with ASD receiving a 16-week course of PRT. Neural changes were specific to N170 latency and were not observed in N170 amplitude or P100 latency and amplitude. This pattern of results suggests that face processing efficiency, rather than basic visual processing of low-level features of visual stimuli, was selectively impacted by PRT. As predicted based on extensive prior evidence, PRT treatment was associated with reductions in autism symptomatology, paralleling changes observed in N170 latency. In our small sample, correlations between magnitude of neural and behavioral change were not observed. Exploratory analyses in subgroups suggest stability of these changes in N170 latency during a 16-week follow-up period after treatment. Similarly, the meaningfulness of changes observed during treatment is supported by stability in the 16 weeks preceding treatment. This pattern of results suggests repeated administration of the experimental assay alone does not lead to N170 change.

These findings offer suggestive evidence of the potential of the N170 as a biomarker sensitive to change in clinical status in the context of intervention. This is an important prospect in several regards. These findings align with prior results using fMRI (12); by extending these findings to EEG, we demonstrate the potential utility of a more economical, scalable, developmentally accessible, and tolerable technology (23) for quantifying neural change in response to treatment. The potential value of a direct measurement of central nervous system change in treatment is significant. All treatments, behavioral or pharmacological, necessarily exert their actions on the brain; objective quantifications of change at the neural level hold potentially greater sensitivity than subjective clinical measures of downstream behavior. In this way, biomarkers could indicate effectiveness in a shorter time scale or with greater sensitivity than the caregiver and clinician rating scales that represent the *status quo* (24).

Limitations and Future Directions

This exploratory study has significant limitations, most notably its small size and cognitively-able sample. This participant profile limits the generalizability of our findings to the ASD

community at large, but the detection of significant effects despite limited statistical power is salient and suggests the value of replication in larger, heterogeneous samples. Though we observed sensitivity to change that paralleled behavioral change, change in biomarker values did not correlate with clinical change. Such correlations would provide stronger evidence of convergent validity and should be re-examined in larger samples with a potentially greater range of change, more granular content in clinical measures, and inclusion of behavioral metrics of face perception. Additionally, this study did not include a non-face stimulus to establish the specifity of effects to social perception. Although this was purposeful to maximize tolerability of the paradigm for young children with ASD, future studies should evaluate the possibility that behavioral intervention improves non-social aspects of visual perception. We note that the absence of observed change at the P100 is supportive of our interpretation of the treatment effects being specifically relevant to social perception. Though we included an attention task and had a behavioral assistant monitor participant attention, rigor would be enhanced by future studies including eye tracking to monitor attention.

These preliminary findings suggest the value of continued investigation of the potential of the N170 as a biomarker in contexts of use related to quantification of treatment response.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Human Investigation Committee at Yale School of Medicine consistent with the 1964 Declaration of Helsinki. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

JM, AN, KP, and PV contributed to study conception and design. EEG acquisition was performed by MR and other members of JM's research team. EEG analysis was completed by MR. MR, DT, AN, and SK performed statistical analyses. PV and her team administered intervention. The manuscript was written by SK and MR. All authors contributed to and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpsyt.2021.709382/full#supplementary-material

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STUDY PROTOCOL
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A Phase II Randomized, Double-Blind, Placebo-Controlled Study of the Efficacy, Safety, and Tolerability of Arbaclofen Administered for the Treatment of Social Function in Children and Adolescents With Autism Spectrum Disorders: Study Protocol for AIMS-2-TRIALS-CT1

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Background: Autism Spectrum Disorder (ASD or autism) is characterized by difficulties in social communication and interaction, which negatively impact on individuals and their families' quality of life. Currently no pharmacological interventions have been shown to be effective for improving social communication in autism. Previous trials have indicated the potential of arbaclofen for improving social function among autistic children and adolescents with fluent speech. The AIMS2TRIALS-Clinical Trial 1 (AIMS-CT1) will examine whether arbaclofen is superior to placebo in improving social function and other secondary outcomes over 16 weeks, along with safety and tolerability profiles.

Methods: AIMS-CT1 is an international, multi-site, double-blind, parallel group Phase II randomized clinical trial. It will include 130 males and females aged 5:0–17:11 years, with a diagnosis of ASD and fluent speech. Eligible participants will be randomized on a ratio of

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1:1 for a 16-week treatment period. Medication will be titrated over 5 weeks. The primary outcome is the effect on social function from weeks 0 to 16 measured on the Socialization domain of the Vineland Adaptive Behavior Scales, 3rd edition™. Secondary outcome measures include the CGI–S (Clinical Global Impression–Severity), CGI–I (Clinical Global Impression–Improvement), other areas of adaptive function, social communication and other autism symptoms, co-occurring behavior problems and health-related quality of life. Genetic and electrophysiological markers will be examined as potential stratifiers for treatment response. Exploratory novel digital technologies will also be used to measure change, examining simultaneously the validity of digital biomarkers in natural environments. The safety and tolerability of the drug will also be examined. Our protocol is very closely aligned with a parallel Canadian trial of 90 participants (ARBA Study, US NCT number: NCT03887676) to allow for secondary combined analyses. Outcomes will be compared using both an Intent-to-reat and Per Protocol approach.

Discussion: The outcomes of this trial, combined with the parallel Canadian trial, will contribute to the evidence base for medications used to help social difficulties among young autistic individuals; demonstrate the capabilities of the AIMS-2-TRIALS network of academic centers to deliver clinical trials; and support future drug development.

Clinical Trial Registration: EudraCT number: 2018-000942-21 and ClinicalTrials.gov registry number: NCT03682978. Currently under protocol v.7.2, dated 20.11.2020.

Keywords: autism, arbaclofen, social function, randomized controlled trial, children, adolescent

BACKGROUND

Autism Spectrum Disorder (ASD or autism) is a highly heritable disorder with prevalence rates in childhood between 1 and 2% (1, 2). Clinical presentation of autistic symptoms typically becomes apparent in early childhood and includes impairments in social interaction and communication, and the presence of sensory anomalies and repetitive and stereotyped behaviors [DSM-5; (3)]. Clinical presentation is highly heterogeneous, depending mainly on age, language, global cognitive levels and accompanying behavioral and/or emotional difficulties (4). A coherent understanding of the neurobiology of autism has not yet been achieved (5). However, recent findings suggest that autism results, in most cases, from atypical neurodevelopmental processes that have their onset *in utero*, and on-going physiopathological mechanisms at molecular, cellular, and circuit levels (6).

The heterogeneity of ASD is not only great at the level of the clinical phenotype but also at the level of the underlying neurobiological mechanisms that may lead to diverse phenotypes. Therefore, different molecules will likely improve deviant behaviors in different subgroups of patients. The study of pathogenic *de novo* mutations in large consortia has led to the identification of several different biological mechanisms as particularly relevant in the etiology of ASD. The study of differential neuropathological pathways that lead to distinct phenotypes and the possibility of finding biomarkers that may index differential pathways or disease trajectories within ASD is one of the most prominent topics in the study of the etiology of ASD and one included in the main objectives of the AIMS-2 TRIALS network (https://www.aims-2-trials.eu/). AIMS-2-TRIALS (Autism Innovative Medicine Studies-2-Trials) began in June 2018 and will run until May 2023. Its purpose is to accelerate medicine development, in particular, precision medicine, via a network of connected scientists and stakeholders across Europe and beyond. The research programme includes a range of work packages and sub-studies led by different academic, scientific and industry professionals to explore how autism develops, from before birth to adulthood, and how this varies in this population. The main aim of the consortium is to study biological

Abbreviations: ABC-C, Aberrant Behavior Checklist-Community version; ADHD, Attention-Deficit/Hyperactivity Disorder; ADOS−2, Autism Diagnostic Observation Schedule, 2nd Edition; AE, Adverse Event; AIM, Autism Impact Measure; ANCOVA, Analysis of Covariance; ASD, Autism Spectrum Disorder; BOSCC, Brief Observation of Social Communication Change; CBCL, Child Behavior Checklist; CGI–I, Clinical Global Impression – Improvement; CGI–S, Clinical Global Impression – Severity; C-SSRS, Columbia-Suicide Severity Rating Scale; CONSORT, Consolidated Standards of Reporting Trials; DMC, Data Monitoring Committee; DSM-5, Diagnostic and Statistical Manual of Mental Disorders, 5th Edition; ECG, Electrocardiogram; EEG, Electroencephalogram; E-I, Excitatory/Inhibitory; ESS-CHAD, Epworth Sleepiness Scale for Children and Adolescents; FXS, Fragile X Syndrome; GABA, Gamma-Aminobutyric Acid; GABAB, GABA type B; IRB/IEC, Institutional Review Board/Independent Ethics Committee; ITT, Intent-to-Treat; IWRS, Interactive Web Response System; PedsQL, Pediatric Quality of Life Inventory; POND, Province of Ontario Neurodevelopmental Disorders Network; PP, Per Protocol; PRN, Pro Re Nata; SAE, Serious Adverse Event; SAP, Statistical Analysis Plan; SCQ, Social Communication Questionnaire; SMURF, Safety Monitoring Uniform Report Form; SRS−2, Social Responsiveness Scale, 2nd edition; SUSAR, Suspected, Unexpected Serious Adverse Reactions; UMCU, University Medical Center Utrecht; VABS−2, Vineland Adaptive Behavior Scales, 2nd Edition; VABS−3, Vineland Adaptive Behavior Scales, 3rd Edition.

markers and relate them to specific phenotypes, which could ultimately benefit from tailored treatments. The consortium will test, in specific subpopulations, new and repurposed medicines to help with social difficulties, repetitive behaviors and sensory processing. Currently, evidence-supported treatment options for core symptoms of autism do not include any pharmacological intervention. The clinical and biological heterogeneity of ASD may partially underlie the lack of positive results in clinical trials. Attention has turned recently to mechanistically targeted treatments. Among the most evidence-supported mechanisms is an excitatory-inhibitory unbalance that affects in, as yet unknown way, the proper coordination of the GABA and glutamate action at the appropriate developmental stages (7).

Glutamate is the most prevalent excitatory neurotransmitter, while gamma-aminobutyric acid (GABA) is the most prevalent inhibitory neurotransmitter in the human brain. Disruptions in GABA-ergic or glutamate signaling have been associated also with other neurodevelopmental disorders including autism and Fragile X Syndrome (FXS), as well as epilepsy (8, 9). A disruption in the excitatory/inhibitory (E-I) ratio is proposed to characterize the autistic brain (9, 10), but the direction of any such imbalance is less clear (11). Dysfunction in GABA signaling has been related to autism-like stereotypies (12). Arbaclofen has been tested in three moderately-sized studies: one in autism, and two in fragile X syndrome. New biomarker investigations have shown that arbaclofen modulates binocular rivalry, which was previously found to be abnormal in autism (13). In healthy controls, they found that arbaclofen increased perceptual suppression relative to placebo, consistent with the understanding of excitatory:inhibitory dynamics in the visual circuits for binocular rivalry. McAlonan et al. (14) very recently reported that arbaclofen dose-dependently rescues differences in visual contrast perception in adults with autism. These psychophysical effects were accompanied by changes in functional connectivity in multiple cortical circuits.

Taking all this evidence together it seems promising to explore medications that target E-I imbalance. Indeed, previous studies with GABA modulators have shown the potential for improving core autistic symptoms. For example, a recent study from the EU-AIMS consortium reported that differences in E-I balance can be 'shifted' using a GABA acting drug (riluzole), and that abnormalities in functional connectivity can be "normalized" by targeting E-I, including autistic adults (15).

One particularly fruitful target may be GABA type B receptors (GABAB). GABAB are crucial for maintaining the E-I balance and pervasive defects in GABAB receptor expression and activity have been associated with autism and are postulated to contribute to co-morbid seizure activity and cognitive impairment (8).

Arbaclofen (previously known as STX209) is a selective GABAB receptor agonist that augments GABA-ergic activity, inhibits presynaptic release of glutamate, inhibits postsynaptic transmission, and modulates intracellular signaling (16–18). Arbaclofen is the active enantiomer of racemic baclofen, an EMA and FDA approved GABAB agonist for spasticity. Baclofen has demonstrated efficacy in treating hyperactivity and audiogenic seizure phenotypes in the fragile X knockout mouse (19, 20). FXS mice models have been found to exhibit deficient GABA-mediated inhibitory neurotransmission particularly notable in the amygdala (21) brain region associated with affective behaviors involving emotional understanding and social interaction. Through elevation of GABA-ergic inhibitory activity, arbaclofen might alleviate autistic symptoms associated with social anxiety and emotional hyperarousal.

A previous trial has investigated whether Arbaclofen improves social difficulties in autism. Seaside Therapeutics initially conducted an open-label, flexible-dose, 8-week Phase II trial of arbaclofen in 32 autistic children and adolescents. Participants were treated with up to 10 mg thrice a day of arbaclofen and reported broad beneficial effects on autistic symptoms with no significant safety or tolerability concerns (22). This study was followed by a randomized, double-blind, placebo-controlled Phase II trial of arbaclofen in 150 autistic individuals between 5 and 21 years of age (23). Following the 12 weeks treatment, participants on arbaclofen and placebo showed no difference on the primary outcome measure [Social Withdrawal/Lethargy subscale of the Aberrant Behavior Checklist; (24)], but showed a nominally significant advantage on the Clinical Global Impression – Severity (CGI–S; 24, 25) for the arbaclofen group. *Post-hoc* exploration showed that drug-related improvements tended to be greater among the more verbally fluent individuals; and per-protocol analysis revealed a nominally significant improvement in of the Socialization domain of the Vineland Adaptive Behavior Scales, 2nd edition [VABS−2; (25)] and when scored by the same clinician both pre- and post-intervention, as per protocol. Safety results showed generally good tolerability, with somnolence and affect lability being more frequent in the active arm of the study. Given the results shown, and in view of the high heterogeneity of the condition that may be masking overall group results, there is rationale to investigate whether more specific, targeted and homogeneous groups (i.e., verbally fluent individuals) may benefit from the treatment with arbaclofen. Therefore, even though with the evidence available so far it is impossible to disentangle whether the positive results on verbally fluent children, adolescents and adults are due to the age and/or language level of the individuals in this group or whether it is the appropriateness of the assessment instruments used, we built on secondary outcome analyses from the Seaside study, and a homogeneous group of verbally fluent individuals was the target of the current clinical trial.

OBJECTIVES

The primary objective of AIMS-CT1 is to examine the effect of arbaclofen vs. placebo on social function and behavior, as assessed through the Socialization Domain of the Vineland Adaptive Behavior Scales, 3rd edition [VABS−3; (26)]. We hypothesize that arbaclofen will be superior in improving social function impairments when compared to placebo. The key secondary objective is to examine the effect of arbaclofen vs. placebo on global functioning, as measured by CGI-I. Other secondary objectives are to examine the effects on other areas of adaptive function (communication and daily living skills), social communication behavior and other autistic symptoms,

co-occurring behavior problems and health-related quality of life. The safety and tolerability of arbaclofen vs. placebo will also be examined. An exploratory objective is to examine whether electrophysiology and sensory discrimination is associated with treatment response to obtain pilot evidence for a predictive biomarker for future trials. An optional DNA sample (with an specific informed consent form) may help us to identify possible response genetic markers. Furthermore, this study adds the use of a novel, exploratory digital biomarker component that will collect data in naturalistic settings with minimal burden for the families. The use of digital biomarkers aims to gather more objective measures of social interaction and understanding than caregivers- or self- reports. They consist of active tasks (social games on a smartphone), passive monitoring of daily behaviors and surveys to participants and caregivers.

METHODS

Trial Design

The AIMS-CT1 is an international, multi-site, double-blind, parallel group randomized placebo-controlled Phase II trial. An Autism Representatives Group created in collaboration with Autistica's DISCOVER Research Network participated in several meetings along the trial and read and gave advice and opinion to the relevant final documents before these were submitted to the Regulatory Agencies. The study will examine the superiority of arbaclofen vs. placebo on the primary and secondary outcomes, along with safety and tolerability profiles over 16 weeks. Participants ($N = 130$) will be allocated to arbaclofen or placebo on a ratio of 1:1 and recruited across seven academic sites in Spain, UK and France. The trial design represents a refinement of the previous study of arbaclofen in ASD (23) and is summarized in **Figure 1**. In collaboration with the Province of Ontario Neurodevelopmental Disorders Network (POND) our protocol is very closely aligned with a parallel Canadian trial of 90 participants (ARBA Study: NCT03887676) for subsequent combination of data.

Participants

Inclusion Criteria

- Male and female participants aged between 5:0 and 17:11 years at time of consent, with a diagnosis of ASD according to the DSM-5 (3) criteria and complex verbal language [defined as qualifying for an Autism Diagnostic Observation Schedule−2 (ADOS−2) Module 3 or 4 assessment (27), as determined by a clinically-certified ADOS-2 rater supervised by the local research-reliable ADOS-2 lead (see Requirements in **Additional Material 5**)].
- The parent/carer (hereafter parent; for brevity) can speak and understand the local language.
- The participant resides with the parent who will complete the primary outcome.
- Willingness to comply with the medication and research protocols.
- Pharmacological treatment affecting behavior stable for at least 6 weeks prior to screening, with no planned changes for the duration of the trial.
- Psychotherapeutic/psychosocial interventions affecting behavior stable for at least 3 months prior to screening, with no planned changes for the duration of the trial.
- Seizure history stable with anticonvulsant medication and seizure free for at least 6 months prior to screening. If anticonvulsant medication has not been stable for at least 3 months, the participant must be seizure free for at least 3 years prior to screening.
- Negative pregnancy test for female participants of childbearing potential.
- Female participants, and female partners of male participants, who are of childbearing potential and sexually active must agree to use highly effective forms of contraception.
- Participant able to take medication orally.

Exclusion Criteria

- Any medical condition that may interfere with the conduct of the trial, confound interpretation or endanger the participant's well-being. This includes but is not limited to impairment of renal function, evidence or history of malignancy, any significant hematological, endocrine, respiratory, hepatic, cardiovascular or gastrointestinal disease, or any clinically significant abnormalities on the electrocardiogram (ECG).
- Prohibited concomitant medications are those that include GABA agonists or modulators [e.g., vigabatrin, tiagapine, clobazam, regular benzodiazepine use (prn and hs use allowed), riluzole].
- Participants who have taken another investigational medicinal product in the 30 days prior to screening or have been involved in a previous trial of arbaclofen.
- A history of hypersensitivity to racemic baclofen.
- Rare hereditary problems of galactose intolerance, the lactase deficiency or glucose-galactose malabsorption.
- Porphyria.
- Active peptic ulceration.
- Engagement in illicit substance or alcohol abuse, according to DSM-5 criteria.
- Breastfeeding females.

Written informed consent will be signed by the participant's parent/carer/legal guardian. The participant will sign the informed consent form or assent form, following local laws and regulations.

Interventions

Arbaclofen manufactured for this trial comes as round white orally disintegrating strawberry-flavored tablets with beveled edges, in strengths of 5, 10, 15, and 20 mg. It is stored at room temperature and will be packed in a box in blister cards of seven tablets. A flexible dose titration schedule will be utilized during the first 5 weeks of treatment (see **Table 1**). Dosing will be stratified by age. For participants aged 5–11 years at time of consent, the starting dose will be 5 mg once daily

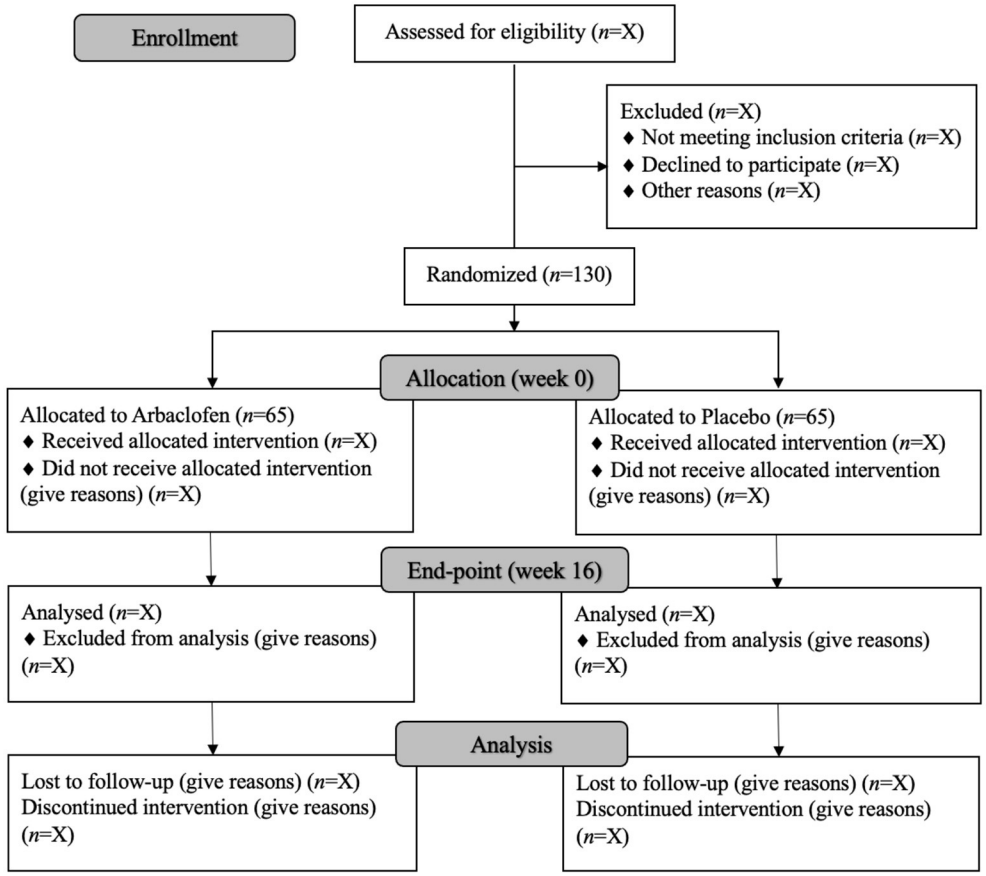

FIGURE 1 | CONSORT diagram showing trial and participant flow through the trial (based on CONSORT guidelines).

and increased to a maximum of 15 mg three times a day. For participants aged 12–17 years at consent, the starting dose will be 5 mg twice a day and increased to a maximum of 20 mg three times a day. Dosing titration is similar to Veenstra-VanderWeele et al. (23) with the addition of one higher dose to be tested in adolescents. If a participant does not tolerate a dose increase, under clinicians' advice, he or she should return to the previous dose level and remain at that level for the remainder of treatment. No change will be made to dosing after the 5-week titration period, unless for safety reasons. The treatment period of 16 weeks is 33% longer than in the study of Veenstra-VanderWeele et al. (23), in which the numerical difference between arbaclofen and placebo groups on the CGI-S had not plateaued at 12 weeks. At the end of treatment, the study medication will be tapered down over a period of up to 13 days.

The comparator is a placebo tablet. The placebo tablets will have a similar shape, mass, color, smell and taste to the arbaclofen tablets and be provided in identical blister cards of seven tablets in an identical box. The placebo tablets will be administered at the same frequency as described above for arbaclofen and stratified by age. The packs will be released to a blinded study team member for dispensing.

Randomization and Allocation

Randomization of participants will be performed 1:1 into arbaclofen vs. placebo by a randomization website (Interactive Web Response System—IWRS) developed by the Data Management Department of the Julius Center, University Medical Center Utrecht (UMCU). Allocation will be sent to an unblinded dedicated pharmacist at each individual site as specified in a separate Pharmacy Manual. A central unblinded monitor will double check dispensation regularly. The rest of the study members are blind to allocation. Randomization will be stratified by site and age group (5–11 years old; 12–17 years old). Unblinding will only happen if the information can help treat an adverse event and for safety reasons.

Outcomes

Table 2 describes all outcomes, safety and sample characterization measures used in the trial. Following *post-hoc* findings in Veenstra-VanderWeele et al. (23), the primary outcome measure chosen is social function as measured by the Vineland Adaptive Behavior Scales, Third Edition (VABS-3)–socialization domain (26) administered by the same rater along the trial, preferably blinded to other outcome measures within a given patient.

TABLE 1 | Dosing and tapering down regime for arbaclofen stratified by age.

Timepoint	Age of participant			
	5–11 years		12–17 years	
	Strength	Frequency of administration	Strength	Frequency of administration
Week 1 (Day 1, V1)	5 mg	Once daily	5 mg	Twice daily
Week 2 (Day 8)	5 mg	Twice daily	10 mg	Twice daily
Week 3 (Day 15, V2)	10 mg	Twice daily	10 mg	Three times a day
Week 4 (Day 22)	10 mg	Three times a day	15 mg	Three times a day
Weeks 5–16 (Day 29+, V3)	15 mg	Three times a day	20 mg	Three times a day
Tapering down regime for those on maximum dose at week 16, V7				
Days 113–116, (V7+3 days)	10 mg	Three times a day	15 mg	Three times a day
Day 117–119	10 mg	Twice daily	10 mg	Three times a day
Day 120–122	5 mg	Twice daily	10 mg	Twice daily
Day 123–125	5 mg	Once daily	5 mg	Twice daily
Day 126	None	–	None	–

V, Visit.

Sample Size

Based on Veenstra-VanderWeele et al. (23), for a 5.3 (standard deviation of 15) change from weeks 0 to 16 in scores on the VABS−3 Socialization domain for those treated with arbaclofen vs. placebo, 100 participants are needed in each arm, assuming 2-sided testing at a significant level of 5 and 80% power. Allowing for 10% attrition, the total sample required would be 220, 110 in each treatment arm. Therefore, the sample from this study will be combined with that of the Canadian ARBA trial to attempt to answer to the key objective. AIMS-CT1 will recruit 130 participants across the seven study sites (i.e., ∼19 participants each) and ARBA-Brain Canada will recruit 90 participants.

Recruitment

Recruitment of participants will be through referral via local autism diagnostic teams and pediatric and Child and Adolescent Mental Health Services at each study site. Consent databases/registers, support groups and advertisements on relevant websites may also be used. Potential participants can also self-refer. After initial contact by attending physician, and provided the participant/legal tutor give consent to be contacted, a study member will contact with the parent/carer and potential participant for pre-screening for eligibility, and, if appropriate, families will be invited to attend a screening visit to be assessed for eligibility and discuss consent. Principal investigator or delegated research team physicians will explain and subsequently obtain informed consent/assent. If patient/family agrees to participate, a schedule for the whole trial will be agreed with them, in order to accommodate families' needs and availability and make the completion of the trial more plausible. Privacy laws and regulations will be adhered to during all procedures related to this study. The collection and processing of participants' personal information will be limited to what is necessary to insure the study's scientific practicability. The local investigator or her/his co-workers will collect data and transfer it without recording the patient's name or date of birth coded with a patient identification number. A patient identification code list linking the individual patients to the identification numbers will be kept at the site; access is restricted to authorized study team members.

Assessments

Table 3 shows the schedule for enrolment, treatment and the visits for participants, including all the assessments conducted at each visit. Participation in the trial will consist of a screening visit followed by eight visits for a period of 18 weeks. **Table 3** shows an overview of the timeline and specific assessments conducted at each visit. To check the adherence, the left over pills get accounted for at all visits and crosschecked with the compliance calendar for medicine intake given to the families.

The trial includes a targeted electroencephalogram (EEG) battery designed to capture sensitive and predictive biomarkers of treatment efficacy at a level that is putatively closer to the underlying neural systems affected by arbaclofen. EEG measures the coordinated electrical activity of pyramidal cells in the outer cortical layers (40).

The task battery (see **Additional Material 1** for full information) is designed to tap potential electrophysiological effects of arbaclofen on the (1) excitatory-inhibitory balance of cortical neural activity, and (2) brain specialization for social processing that may relate to the social functioning targeted in the trial, manifested by brain responses to social stimuli (41, 42).

An optional blood sample for DNA extracting is collected from participants and his/her parents that specifically consent to this. Samples are then sent for analyses to Institut Pasteur (Paris, France) where is and safely stored for 25 years after the explicit consent of the patient/legal/parents with a personal code with no connection with any personal information. Also optional, is the inclusion of digital biomarkers (see **Additional Material 1**), to obtain pilot evidence for treatment responsive biomarkers relevant to core and associated symptoms of autism.

TABLE 2 | List of study measures.

Name of measure	Measure details
Primary outcome	
Socialization domain of the Vineland Adaptive Behavior Scales, 3rd edition (VABS−3) (26)	The Socialization Domain of the VABS−3 measures social function and behavior, covering interpersonal relationships, play and leisure and coping skills. The informant (parent/carer) is required to reside with the participant. The same informant and interviewer will be used at both timepoints, and any change will be reported as a protocol deviation, and wherever possible the interviewer will be blind to other study assessments. Interviewers administering the VABS−3 will be required to achieve 90% reliability with a gold standard rater in order to administer it for the trial. Regular reliability meetings will be scheduled for VABS−3 gold standard raters and interviewers across study sites to maintain reliability.
Key secondary outcomes	
Clinical Global Impression – Severity (CGI–S) scale (28, 29)	The CGI–S will assess the severity of impairment in global functioning, including but not limited to social engagement, internalizing and externalizing problems. It is rated on a 7-point scale ranging from 1 (normal, not at all impaired) to 7 (among the most extremely impaired) by a treating clinician. A CGI-S score of 3 (mildly impaired) will be the anchor applied to all participants meeting diagnostic criteria for ASD, with higher scores indicating significant co-occurring problems.
Clinical Global Impression – Improvement (CGI–I) scale (28, 29)	The Clinical Global Impression – Improvement (CGI–I) scale will measure improvement in global functioning during the previous week since treatment initiation. It will be rated by a treating clinician on a 7-point scale ranging from 1 (very much improved) to 7 (very much worse), with 4 representing no change. All available information will be used to inform clinical judgement. The CGI–I scores will also be used to assess safety, with participants who have CGI–I scores of 6 or more (much or very much worse) for two or more consecutive visits being discontinued from the trial.
Other secondary outcomes	
Communication and Daily Living Skills domain of the Vineland Adaptive Behavior Scales, 3rd edition (VABS−3) (26).	The Communication and Daily Living Skills domains of the VABS-3 (described in primary outcome above) will be administered to assess these areas of adaptive behavior.
Brief Observation of Social Communication Change (BOSCC) (30)	The BOSCC is a brief, 12-min observation of a semi-structured social interaction between the participant and an examiner. The interaction is video-recorded, and autism symptoms are coded using an algorithm.
Social Responsiveness Scale, 2nd edition (SRS−2) (31)	The SRS−2 is a 65-item measure identifying the presence and severity of autistic symptoms. Items are rated on a 4-point scale with higher scores reflecting more severe autism. It will be completed by parents/carers and teachers. The teacher completing this measure should spend at least 10 h per week in direct contact with the participant.
Autism Impact Measure (AIM) (32)	The AIM will be completed by the parent/carer to assess symptoms of autism. It consists of 41 items measuring both the frequency and impact of autism symptoms using a 5-point scale.
Aberrant Behavior Checklist - Community version (ABC-C) (24)	The ABC-C will also be completed by the parent/carer to measure irritability, lethargy/social withdrawal, stereotypic behavior, hyperactivity/non-compliance, and inappropriate speech displayed by the participant. Fifty-eight items are rated on a 4-point scale, with higher scores indicating more severe problem behaviors. The ABC-C will be completed by the parent/carer.
Child Behavior Checklist (CBCL) (33)	The CBCL will be used to measure emotional and behavioral problems. Items are rated on a 3-point scale with higher scores indicating more problems. Two different versions will be administered to parents of 5-year olds (1–5-year version) and 6–17-year olds (6–18-year version).
Pediatric Quality of Life Inventory (PedsQL) (34)	The PedsQL measures health related quality of life in children and adolescents. The Generic Core Scales, consisting of 23 items rated on a 5-point scale, will be used to measure physical and psychosocial health. Parents/carers will complete one of three different versions dependent on the participant's chronological age (5–7; 8–12; 13–18 years).
Exploratory measures	
Electrophysiology and sensory processing	Electrophysiology and sensory processing will be measured using electroencephalograms (EEG). Computer tasks chosen specifically for the trial will be completed whilst the EEG is being conducted to measure resting state, response to social and non-social stimuli, auditory processing and habituation response. The tasks take about 1 h to complete.
DNA acquisition	DNA samples (using 6 mls EDTA tubes) will be obtained from patient, and both parents when possible. This will help us to explore the genetics of those responders to the drug, vs. non-responders.
Digital Biomarkers	Digital biomarker (dBM) technology allows the remote measurement of the signs and symptoms of ASD, which can potentially reduce the burden of site visits and allow frequent/daily tracking in an ecologically valid environment. Patients will be asked to complete some tasks on a pre-set mobile phone and wear a smart wrist watch during the time of the study. Bluetooth beacons will be used to estimate the frequency of social interactions at home.
Safety assessments	
Medical checks	Vital signs (pulse, temperature and non-supine blood pressure) will be checked by a treating clinician. Physical examinations and electrocardiograms will be performed, and height and weight will be recorded with all outer wear and shoes removed. Safety blood tests checking complete blood count, liver enzymes, renal function and non-fasting glucose will be performed. Pregnancy testing will be performed for female participants of childbearing potential. Drug testing will also be performed on urine samples.
Safety Monitoring Uniform Report Form (SMURF) (35)	The SMURF will be administered by a treating clinician to record possible adverse events of psychotropic medication. Where appropriate, the participant and their parent/carer will be interviewed together and events since the last visit will be sought.
Epworth Sleepiness Scale for Children and Adolescents (ESS-CHAD) (36)	The ESS-CHAD will be administered by a treating clinician to measure daytime sleepiness and sedation in the past week. It consists of 8 items tapping into different situations which are rated on a 4-point scale with higher scores indicating greater chance of sleepiness.

(Continued)

TABLE 2 | Continued

Name of measure	Measure details
Columbia-Suicide Severity Rating Scale (C-SSRS) (37)	Suicidality assessments will be completed by a treating clinician using the C-SSRS to measure the presence and intensity of suicidal ideation and behavior. The "baseline" version will be administered at week 0 and the "since last time" version thereafter. Two different versions will be administered depending on the chronological age of the participant.
Screening/characterization assessments	
Autism Diagnostic Observation Schedule−2 (ADOS−2) (27)	The ADOS−2 modules 3 or 4 will be administered to enable characterization of autism symptoms and severity. If a reliable ADOS−2 assessment has been administered in the 24 months prior to screening, with family consent, the scores from the previous assessments will be used an administration of these assessment will not be required.
Wechsler Scales	The appropriate Wechsler Scales according to age will be administered to enable characterization of the cognitive functioning of the sample. If a reliable standardized cognitive assessment has been administered in the 24 months prior to screening, with family consent, the scores from the previous assessments will be used an administration of these measures will not be required.
Social Communication Questionnaire-Lifetime version (SCQ) (38)	The SCQ is a 40-item yes-no measure of autism symptoms and severity and will be completed by the parent/carer. Scores of 15 or greater indicate a possible ASD.
Repetitive Behavior Scale-Revised (RBS-R) (39)	This questionnaire is part of the digital biomarkers optional sub-study and only completed if opted in. The RBS-R is an empirically-derived comprehensive survey of the entire spectrum of repetitive behaviors clinically observed and referred to in the DSM-IV (3) diagnostic description of Autistic Disorder. Parents or caregivers rate 43 behaviors on a scale of 0–3, where 0 indicates the behavior does not occur and 3 indicates the behavior does occur and is a severe problem.

All the procedures undertaken and all relevant clinical information is collected both in the electronic CRF and the electronic medical record.

Statistical Analysis

Analysis will be performed both with an Intent to Treat (ITT) and as Per Protocol (PP) approach. The initial efficacy analysis will use the ITT sample, which includes all participants randomized who have at least one dose of medication and one post-baseline assessment. To be included in the PP sample, participants will fulfill ITT criteria and have attended at least 80% of the visits, have taken at least 70% of the prescribed medication and have a consistent pre- and post-intervention VABS−3 clinician rater.

Efficacy will be indicated by a difference between treatment arms at week 16, using Analysis of Covariance (ANCOVA; with follow-up score used as the dependent variable and treatment group as a factor) or logistic regression, and Chi-Square techniques, as appropriate. Age will be adjusted for and baseline scores will be used as covariates. Hypothesis testing will be performed at the 5% level of significance for 2-sided tests. Safety analyses will be conducted on all participants taking at least one dose of study medication by calculating the incidence of adverse events in each arm and summarizing laboratory and ECG assessments, physical examinations and vital signs.

A secondary analysis will be conducted by combining data from AIMS-CT1 and the ARBA Study to ensure statistical power for the assessment of efficacy of the primary outcome variable. Additional analyses will consider variables or sub-variables that will be different from or derivative of the primary and secondary hypotheses. Another level of analysis will look at data of individual sites. Power calculation was made for the primary outcome (VABS-3 social function domain), which justifies the a priori planned combination of the data of this study with that of ARBA Study in Canada. Other results will be considered secondary results and therefore more exploratory and hypotheses-generating.

While no interim analysis is planned, if necessary, the Data Monitoring Committee (DMC) will consider it and inform the Sponsor whether premature termination criteria are met.

Adverse Event Reporting

A medical history and physical examination will be conducted at screening to assist with interpretation of any adverse events (AEs), whether serious (SAEs) or non-serious, that occur during participation in the trial. The standard definition of AEs and SAEs will apply. In addition, pregnancy (in participant, or female partners of male participants), overdose (either accidental or intentional), cancer, potential drug-induced liver injury and suspected transmission of an infectious agent will be recorded as SAEs and follow these reporting regulations. In all cases of pregnancy, the treatment will be permanently discontinued in an appropriate manner and follow-up information on the course of pregnancy should be recorded if permission is given by the participant.

AEs and SAEs may be reported by the participant and/or their parent or found through medical examination or laboratory testing during phone call monitoring or face-to-face visits. The nature of each event, onset, duration and severity will be established and recorded, regardless of whether the event is related to the study treatment or not. AEs will be recorded from the initiation of study treatment until the final visit. All SAEs that occur between screening and up until 30 days of the last dose of the study treatment will be recorded. All AEs and SAEs will be followed-up proactively at subsequent visits/phone calls until either resolution, the condition stabilizes, the event is otherwise explained, or the participant is lost to follow-up. Follow-up is also required for AEs that cause interruption or discontinuation of the study treatment.

TABLE 3 | Participant timeline showing schedule of enrolment, treatment and visits for participants.

		STUDY PERIOD															
		Screening	Baseline	Treatment													Follow-up
Visit		SC	1	PC	2	PC	3	PC	4	PC	5	PC	6	PC	7	8	
Week		1-3	0	1	2	3	4	5	6	7	8	10	12	14	16	18	20
Observation window (days)			+7-21	±4	±4	±4	±4	±4	±4	±4	±4	±4	±4	±4	±7	(V7+14) -1/+4	+14
ELIGIBILITY	Consent form	X															
	Inclusion/exclusion	X															
	ADOS–2 mod3/4	X															
characterization	Wechsler scales	X															
	SCQ		X														
OUTCOME ASSESSMENTS	VABS–3		X												X		
	CGI-S		X		X		X		X		X		X		X	X	
	CGI-I				X		X		X		X		X		X	X	
	BOSSC		X												X		
	SRS–2 (Parent & Teacher)		X												X		
	AIM		X				X				X		X		X		
	PedsQL		X				X				X		X		X		
	ABC-C		X				X				X		X		X		
	CBCL		X												X		
	EEG		X												X		
	Digital Biomarkers*	X	X		X		X		X		X		X		X		
	DNA*	X															
SAFETY	Vital signs	X	X		X		X		X		X		X		X	X	
	Physical exam	X	X		X		X		X		X		X		X	X	
	ECG	X													X		
	Safety blood tests	X													X		
	Urinalysis (including drug testing)	X													X		
	Pregnancy testing	X	X (if >7 since SC)				X				X		X		X		
	SMURF		X		X		X		X		X		X		X	X	
	ESS-CHAD		X		X		X		X		X		X		X	X	
	C-SSRS		X		X		X		X		X		X		X	X	
	Concomitant medication		X		X		X		X		X		X		X	X	
	Self-reported AE			X		X		X		X		X		X			X
TREATMENT 1:1	Arbaclofen																
	Placebo																
				Titration period (5 weeks)					Treatment period (11 weeks)							Taper down (2 weeks)	Post-treatment (2 weeks)
ADHERENCE	Medication accountability		X		X		X		X		X		X		X	X	
	Medication compliance diaries			X		X		X		X		X		X			

Columns shaded in gray are the face-to-face visits. ABC-C, Aberrant Behavior Checklist-Community; ADOS−2, Autism Diagnostic Observation Schedule, 2nd edition; AIM, Autism Impact Measure; BOSCC, Brief Observation of Social Communication Change; CBCL, Child Behavior Checklist; CGI-S, Clinical Global Impression-Severity; CGI-I, Clinical Global Impression-Improvement; C-SSRS, Columbia-Suicide Severity Rating Scale; ECG, Electrocardiogram; EEG, Electroencephalogram; ESS-CHAD, Epworth Sleepiness Scale for Children and Adolescents; PC, Phone call; PedsQL, Pediatric Quality of Life Inventory SC, Screen; SCQ, Social Communication Questionnaire; SMURF, Safety Monitoring Uniform Report Form; SRS−2, Social Responsiveness Scale, 2nd edition; VABS−3, Vineland Adaptive Behavior Scales, 3rd edition.

**optional [the digital biomarkers include the completion of one small cognitive task every day, the completion by the parent of the RBS-R questionnaire (39) at visits 1 and 7, and the completion of satisfaction questionaries at visit 7; for DNA, samples will be also collected from both parents if applicable].*

Usual convention will be used for reporting all AEs and SAEs following local laws and regulations. All SAEs will be reported to the sponsor and sponsor delegate within 24 h of the study site becoming aware of the event. Any Suspected, Unexpected Serious Adverse Reactions (SUSARs) will be reported to the appropriate regulatory authorities following local and global guidelines and requirements.

Context

AIMS-2-TRIALS Network

AIMS-CT1 is one of the many studies taking place within the AIMS-2-TRIALS network (www.aims-2-trials.eu), which aims to apply a precision medicine approach to ASD and improve patient outcomes by tailoring treatments to a patient's biological profile. Building on the achievements of other IMI (Innovative Medicines Initiative) initiatives, Horizon 2020 networks, and SMEs (Small Medium Enterprise), the specific objectives are to validate and qualify stratification biomarkers from infancy to adulthood; develop objective outcome measures that can be used in trials; create a European-wide clinical trials network that reliably carries out studies able to support filings to the European Medicines Agency/ Food and Drug Agency (EMA/FDA); to carry out better targeted clinical trials linked to other international efforts—including quick wins or "fast fails" of ineffective agents—and to translate molecular mechanisms and drug effects between preclinical models and particular subtypes of ASD.

COVID Pandemic Adjustments

In March 2020, the WHO declared a COVID-19 global pandemic that would eventually affect health systems all over the globe. The European Medicines Agency (EMA) and the National Regulation Authorities, working with all relevant stakeholders, acknowledged the impact of the pandemic on the conduct of clinical trials and have been issuing additional guidelines for their management. The Sponsor and Principal Investigators of the present clinical trial have taken into account all relevant guidelines and local legislations and have made and reported to the authorities for their approval the appropriate adjustments to the design and conduct of the study. All these adjustments have been included in the updated versions of the Protocol. The most significant ones are: (i) recruitment of new participants was halted during the period that National global lockdowns were in place, (ii) some physical visits were converted into remote visits at the beginning of the pandemic; by phone and/or video-conferencing depending on the assessment instrument

after authors/supervisors of the main evaluation instruments were consulted; in this case, vital signs were recorded at local pharmacies; primary outcome measure (social function by the VABS-3 social domain score) was conducted via videoconference at the time of lockdown or whenever it was more risky for the families to attend the hospital, by the same rater and with the same informant; the same format (video or in-site) was maintained for visit 1 and visit 7 for the same patient (iii) transferring of participants specific assessments (e.g., EEG) to investigational facilities away from risk zones or, if possible, to the participants' homes, (iv) transfer of sample extraction, medication provision (ensuring maintenance of temperature control) and other medical/nursing procedures to authorized local facilities and/or participants' homes if possible, (v) on-site monitoring transferred to hired local monitors (vi) local instructions followed at all times for screening for COVID-19 symptoms and enhanced social distancing and protection and cleaning regimes are in place as per site local instructions. For all decisions, a benefit-risk balance of the integrity of the trial and well-being of the participants was taken into consideration with safety always being the prevailing concern.

DISCUSSION

The AIMS-CT1 is a phase II double-blind, parallel group, randomized placebo-controlled trial designed to investigate the efficacy, safety and tolerability of arbaclofen for social function over 16 weeks in 5 to 17-years-old autistic males and females with fluent speech. It will also examine the superiority of arbaclofen vs. placebo on global functioning, other areas of adaptive functioning, social communication behavior, autistic symptoms, co-occurring behavioral problems and health-related quality of life. The outcomes of this trial, combined with the parallel Canadian ARBA Study, will contribute to the evidence base for medications used to help reduce social difficulties among young autistic individuals. Positive findings have the potential to improve the quality of life for young autistic individuals and those involved in their care. Improvements in social function in the short-term may lead to increased participation in social activities and better outcomes for young people with autism. If arbaclofen is found to improve social function, further trials are warranted, including for other medications targeting excitatory/inhibitory imbalances in autism.

Testing pharmacological treatments for autistic symptoms has been challenging because of the vast variation in phenotypic presentation and limited understanding of underlying causes. A key limitation of trials for autism is the generalizability of any effects of the tested drug to individuals across the whole spectrum, and in the case of this particular trial, specifically to those autistic individuals who have more significant cognitive impairment or less fluent verbal ability. Additional research is required to investigate this, given the additional impacts and costs co-occurring intellectual disability has on the individual, their family and society (43).

Design of the current trial was informed in two important ways by lessons learnt from the Veenstra-VanderWeele et al. (23). First, the primary outcome is the Socialization domain of the VABS−3 (26). Following results in Veenstra-VanderWeele et al. all efforts will be made to maintain the same interviewer and informant at both time points and any change in interviewer or informant will be reported as a protocol violation. When possible, the VABS−3 rater will be blind to other study assessments, otherwise this will be considered a protocol deviation. All interviews will be recorded for external monitoring, with a central gold standard rater per site that will review at least 25% of the recordings and provide feedback to raters. Specific training was provided within the trial team across sites to address the particular challenges of conducting an international study conducted in multiple languages, and the trial team consulted with a co-author (Dr. Celine Saulnier) of the instrument on training on the VABS−3.

Secondly, we employed an inclusion criterion for the participant to be verbally fluent (instead of using an IQ criterion). Previous pharmacological trials in autism have either enrolled participants across a wide range of functional skills, or restricted enrolment to individuals with IQ scores above a certain threshold [e.g., IQ $\geq$ 70; (44)]. The rationale for restrictive enrolment has been that drug effects might only be evident in the higher IQ subgroup. In fact, however, there are no mechanistic biological hypotheses to support such supposition for any drug or drug mechanism. Rather, any apparent disadvantage in response seen in individuals with weaker functional skills is likely related to limitations in outcome assessment for these individuals. Standardized cognitive and behavioral outcome measures often show limited resolution in lower ranges of development, as they are not intended or designed as measures of change in significantly impaired cohorts. *Post-hoc* analyses (unpublished) of the Seaside Therapeutics trial (23) of Arbaclofen showed numerically larger benefit in participants with stronger functional skills, regardless of whether those skills were defined by IQ, verbal fluency (ADOS module 3 or 4, vs. module 1 or 2), or Vineland Communication domain age-equivalent scores. AIMS-CT1 will employ the criterion for verbal fluency based on ADOS−2 module, which aligns with clinically meaningful distinctions in communicative function, rather than employing an arbitrary numerical threshold from some language assessment measure. If treatment results in improved social function, individuals who are verbally fluent will be able to manifest their improvement in communicative behaviors that are evident to their social partners and to observers, including parents, and can be quantified precisely on available psychoeducational measures. Based on our own meta-analyses (45), we included some of the variables (e.g., flexible dosing, reduced number of recruiting sites, threshold of baseline symptoms) to reduce placebo effect.

AIMS-CT1 was initiated prior to the COVID-19 pandemic. The pandemic led to a suspension of recruitment in all sites. In two sites that had commenced enrollment and randomized 14 participants, and in alignment with national and international official and expert-driven guidelines, procedures were adapted to limit face-to-face visits. It is unknown how the COVID-19 pandemic and lockdown policies that change dramatically the routines and interventions received by the participants in the trial and change the way patients are assessed during the trial

will impact on the nature of data collected for these individuals along with future participants and we recognize there may be potential effects that will need to be monitored. However, randomization should mean that these issues will affect each arm equally. All protocol deviations or violations that occurred due to the pandemic obligations have been recorded in detail for report to authorities and to inform the monitoring board of the trial as has been recommended (46, 47). The experience of running a clinical trial through the pandemic has shown the importance of identifying the most important aspects within the trial in which strict procedures need to be followed and other procedures and/or measurements in which flexible acquisition or assessment procedures and their means can be anticipated per protocol. For this, experienced and reliable researchers, as those available in large academic centers, facilitate adaptation to unexpected circumstances.

ETHICS STATEMENT

Ethical approval for the trial has been obtained from: France: Comité de Protection des Personnes (CPP) "Sud-Méditerranée IV" (Ref CPP: 19 09 04), L'Agence Nationale de Sécurité du Médicament et des produits de santé (ANSM, French Medicines Agency); Spain: CEIm Hospital General Universitario Gregorio Marañón, Madrid (Ref: #Cod. CNH: 280246# [Madrid]); Agencia Española del Medicamento y Productos Sanitarios (AEMPS); UK: East Midlands – Leicester Central Research Ethics Committee (Ref: 18/EM/0335) and UK Medicines and Healthcare products Regulatory Agency (MHRA). Written, informed consent will be given by the participant's parent/carer/legal guardian and participant, if applicable, in line with local laws and regulations. Assent from the participant will be obtained in all cases. An independent Data Monitoring Committee (DMC) comprising of expert clinicians and a statistician will provide oversight of the trial conduct and monitor safety, efficacy and dose escalations. DMC members: Professor Alessandro Zuddas, Università di Cagliari, Sardinia, Italy; Professor Benedetto Vitiello, Università degli Studi di Torino, Turin, Italy and John Hopkins University, Baltimore, MD, United States; Dr. Michael McIsaac, University of Prince Edward Island, Prince Edward Island, Canada (Statistician).

AUTHOR'S NOTE

Further information with regards to Trial status, Confidentiality and Dissemination can be found in online **Additional Material 2**, complete SPIRIT ckecklist can be found in **Additional Material 3** and a model of Consent Form in **Additional Material 4**.

AUTHOR CONTRIBUTIONS

CA, MPar, PW, TC, DM, EJ, and EL: conception of the study, protocol writing, and obtained funding. MPar, ASJ, RD, JP, EJ, LM, EA, DM, EL, PW, TC, AS, CC, and TB: contribution to the development and design of the study. MPar, MPal, ASJ, PW, TC, AS, MM, FM, VP, PSh, and PSi: helped to draft the manuscript. All authors read and approved the final manuscript and involved in reviewing and revising the manuscript for content.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpsyt.2021.701729/full#supplementary-material

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The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Investigating Changes in Reward-Related Neural Correlates After PEERS Intervention in Adolescents With ASD: Preliminary Evidence of a "Precision Medicine" Approach

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Background: The Social Motivation Hypothesis proposes that individuals with autism spectrum disorder (ASD) experience social interactions as less rewarding than their neurotypical (TD) peers, which may lead to reduced social initiation. Existing studies of the brain's reward system in individuals with ASD report varied findings for anticipation of and response to social rewards. Given discrepant findings, the anticipation of and response to social rewards should be further evaluated, particularly in the context of intervention outcome. We hypothesized that individual characteristics may help predict neural changes from pre- to post-intervention.

Methods: Thirteen adolescents with ASD received the Program for the Education and Enrichment of Relational Skills (PEERS) intervention for 16 weeks; reward-related EEG was collected before and after intervention. Fourteen TD adolescents were tested at two timepoints but did not receive intervention. Event-related potentials were calculated to measure anticipation of (stimulus-preceding negativity; SPN) and response to (reward-related positivity; RewP) social and non-social rewards. Additionally, measures of social responsiveness, social skills, and intervention-engagement were collected. Group differences were analyzed as well as individual differences using prediction models.

Result: Parent-reported social responsiveness and social skills improved in adolescents with ASD after participation in PEERS. ASD adolescents displayed marginally decreased anticipation of social rewards at post-intervention compared to pre-intervention. Regression models demonstrated that older adolescents and those with *lower* parent-reported social motivation prior to participation in PEERS displayed marginally increased social reward anticipation (more robust SPN) from pre- to post-intervention. Participants who displayed more parent-reported social motivation before intervention and were more actively engaged in the PEERS intervention evidenced increased social reward processing (more robust RewP) from pre- to post-intervention.

Conclusion: Findings suggest that there may be differences in saliency between wanting/anticipating social rewards vs. liking/responding to social rewards in individuals with ASD. Our findings support the hypothesis that identification of individual differences may predict which adolescents are poised to benefit the most from particular interventions. As such, reported findings set the stage for the advancement of "precision medicine." This investigation is a critical step forward in our ability to understand and predict individual response to interventions in individuals with ASD.

Keywords: reward processing, PEERS intervention, autism, social motivation, precision medicine

INTRODUCTION

There is a current lack of universally accepted terminology for describing autism (1) and as such, several terms are used in this paper to describe adolescents with autism. We used both person-first language and identity-first language in an effort to be inclusive of numerous current perspectives on appropriate terminology.

Autism and Social Motivation

Children with autism spectrum disorder (ASD) have reduced preferences toward social information compared to their neurotypical or typically developing (TD) peers (2, 3). The Social Motivation Hypothesis proposes that the brain's reward centers are related to early impairments in social attention due to social stimuli being less rewarding, thus setting a series of negative developmental consequences in motion (4). This may result in a reduction in social orienting, social interaction, and social skills—all of which may lead to broader deficits in social behaviors (4). Demonstration of the social motivation hypothesis often relies on the use of brain-based methods, including neural and neuropsychological markers of reward processing (5). Reward centers of the brain include mesolimbic dopamine system, comprised of the midbrain (via the ventral tegmental area) and striatum (via the nucleus accumbens) (6, 7).

Social Motivation and Neural Response

Though some research suggests that children with ASD have less reward-related brain activity than their neurotypical peers in response to faces (8, 9), other work suggests that individuals with ASD evidence hypoactivity in the reward system in response to all stimulus types (10).

One way to approach mixed findings is by examining differences in reward-related brain activity by evaluating the difference between *anticipating* vs. *processing* rewards. Anticipation is linked to cues of reward and may become reinforced when the reward is more attractive or salient. Similarly, response to reward (i.e., reward processing) is enhanced if the reward is preferred but dampened if the reward is non-preferred. Anticipation of and response to rewards involve separate cognitive processes and both processes should be investigated in order to understand the entirety of how the reward system functions in individuals with and without ASD. Moreover, metrics of anticipation tend to be overlooked in paradigms designed to measure reward processing (11), which may contribute to mixed neural findings. A meta-analysis of functional magnetic resonance imaging (fMRI) studies examining anticipation of and response to rewards suggests that reward differences in ASD may apply to both social and non-social stimuli (12). Specifically, the caudate, nucleus accumbens, and anterior cingulate gyrus were hypoactive during anticipation of and in response to social and non-social rewards (12). These findings expand upon initial theories of disrupted reward systems more broadly.

Electroencephalographic (EEG) methods may serve to further elucidate the complexity of reward processing in ASD, as high temporal resolution is a notable feature and thus complements the high spatial resolution of fMRI. Additionally, EEG is a relatively inexpensive, non-invasive technique that is well-tolerated across the psychiatric spectrum. Using event-related potentials (ERPs), the stimulus-preceding negativity (SPN) component measures brain activity prior to stimulus presentation and may serve as a measure of anticipation. The reward-related positivity (RewP) ERP measures response to rewards and reflects the evaluation of rewards (i.e., determining if a reward is "liked" or "disliked") by comparing losses to gains (13, 14). There is evidence to suggest that the SPN and RewP support the social motivation hypothesis, as children with ASD with less severe social impairments display larger reward anticipation (SPN) (15) and reward response (RewP) to faces (16).

Behavioral Interventions for ASD

Behavioral interventions have been designed to improve social communication skills in ASD—by augmenting interactions with others and helping individuals with ASD form meaningful relationships; for reviews see (17, 18). The Program for the Education and Enrichment of Relational Skills (PEERS) intervention is a manualized, evidence-based group intervention designed to provide adolescents with ASD skills to both make and keep friends; see methods section for additional details (19–21). PEERS is efficacious in increasing social skills, frequency of social get-togethers, and friendships (20, 22).

Objective Outcome Measures for Intervention

Objective measures, including brain-based measures, may identify factors that result in favorable intervention outcomes. To our knowledge, <10 studies have been published using measures of neural response as either an outcome measure or predictor

of response to empirically supported behavioral intervention in individuals with ASD (16, 23–30). Of these studies, four used fMRI, and five used EEG methodology (16, 23, 24, 29, 30). Seven measured brain activity both before and after interventions (16, 24–27, 29, 30), five of which found increased brain activity in response to social stimuli (e.g., while viewing faces or in response to point-light displays of biological motion) (16, 25–27, 29). A majority of these investigations were done in children under 5 years, leaving much to be learned regarding adolescents' neural response to intervention.

As such, there is a pressing need for biomarkers that can detect meaningful intervention outcomes. Biomarkers may also address the heterogeneity of ASD through the identification of homogeneous subgroups of individuals based on biological factors. The N170, a neural measure of face processing and perception, is currently the only psychiatric biomarker for ASD approved by the Food and Drug Administration (31). It has been shown to be a sensitive measure of change due to the effects intervention while also identifying groups of individuals with ASD who have similar pathophysiology (23, 29, 31). Social difficulties in autism are underscored by aberrant processing of social information, as evidenced by a slower response (longer N170 latency) to faces compared to TDs (32–34), including in response to emotional faces (35). Given that the N170 is also closely associated with social communication challenges in ASD, it is a biomarker grounded in core ASD symptomatology.

Use of Neural Response Before and After PEERS

Of the aforementioned papers using measures of neural response as an intervention outcome measure, two looked at brain activity before and after participation in PEERS. Van Hecke et al. measured resting state EEG before and after PEERS (24). The authors found that after participating in PEERS, teens with ASD displayed increased left-dominant gamma asymmetry, such that their brain activity appeared similar to that of neurotypical teens (24). Left-hemisphere dominance is associated with increased motivation and affect, while right-hemisphere dominance is associated with withdrawal and negative emotional style (36, 37). Additionally, Van Hecke et al. (24) found that after intervention, teens with ASD who (a) displayed fewer symptoms of ASD, (b) had more get-togethers with other adolescents during the intervention, and (c) displayed greater understanding of PEERS-specific concepts showed the greatest relative left-hemisphere dominant EEG activity in the gamma band. Therefore, it appears that individual characteristics seem related to the degree of left-dominant pattern of hemispheric asymmetry post-intervention.

In a second investigation of brain activity before and after PEERS (16), there was evidence of enhanced reward processing (as measured by the RewP) in teens with ASD after completion of PEERS. These findings suggest a malleability of social motivation in adolescents with ASD after social skills training. Additionally, the investigators found that adolescents with ASD who displayed less robust social reward processing prior to intervention made the most gains in social responsiveness, social skills, and PEERS-specific knowledge after intervention (16). That is, teens with ASD who displayed *less* response to social rewards prior to PEERS appeared to benefit the most from intervention. Thus, it appears critical to measure the contribution of unique individual factors to identify which individuals stand to benefit the most from intervention.

One such individual factor that remains unexplored is teen engagement in behavioral intervention. Motivation to participate in intervention, by way of active participation within sessions, may predispose adolescents to receive more benefits compared to those who are less engaged. PEERS was originally validated in children and teens ages 11–16 years (22), a developmental period from late childhood through adolescence characterized by increased social demands (33). As such, age should be considered as a potential moderator to the effects of intervention. Age is also relevant in brain-based studies of reward processing, as younger individuals (e.g., early adolescents) with ASD appear to show greater variability in striatal activation during social reward tasks compared to older individuals with ASD, which may contribute to differences in anticipation vs. response processes in ASD (12).

Current Study

The current study, which is a preliminary model of using a "precision medicine" approach to intervention, was designed to answer the following questions:

1. How does reward-related brain activity, both anticipation (SPN) and processing (RewP), to social and non-social stimuli change from pre- to post- PEERS intervention in a sample of adolescents with ASD?
2. How does brain activity related to anticipation of and response to social and non-social rewards differ across time between adolescents with ASD receiving PEERS vs. typically developing (TD) adolescents not receiving PEERS?
3. Does change in reward-related brain activity before and after intervention relate to individual factors? That is, can individual change in reward anticipation and processing from pre- to post- PEERS intervention be predicted by individual characteristics (e.g., age, social skills)?

To our knowledge, this is the first study to: (A) measure electrophysiological correlates of both anticipation of and response to social and non-social stimuli in teens with ASD before and after participation in PEERS, and (B) compare brain activity of teens with ASD before and after PEERS to brain activity of TD teens across time. Exploratory analyses on the N170 were performed after visual inspection of the ERP data; see Methods for details.

METHODS

Participants

Participants included 13 adolescents with ASD and 14 sex-, age-, IQ-, and race-matched TD adolescents; see **Table 1**. A total of 17 ASD participants were initially enrolled in the study. However, four dropped out for reasons including: difficulty with transportation, psychiatric hospitalization, and the adolescent no longer wanting to attend sessions. Thus, 13 ASD participants were included in the final sample. The 14 TD participants were

TABLE 1 | Descriptive characteristics of the autism spectrum disorder (ASD) and neurotypical (TD) groups at Time 1.

Characteristics	ASD $n = 13$	TD $n = 14$
Sex	10 male, 3 female	12 male, 2 female
Age [M (SD), Range]	14.17 (2.09), 11.3–17.1	13.22 (1.63), 11.1–17.1
IQ, M (SD), Range	99.54 (15.62), 77–129	106.14 (15.49), 79–131
Race (*n*)		
White *n*	3	4
Latinx *n*	9	8
Mixed race/other *n*	1	2
Maternal education level (*n*)		
Less than college	10	5
College and above	3	9
Household income (*n*)		
Up to $50,000	4	4
$50, 001–100,000	5	4
Over $100,001	4	5
Missing data	–	1

The ASD and TD samples are well-matched on sex, age, IQ, race, and household income. However, we note that maternal education is lower in the ASD group compared to the TD group.

not enrolled in the PEERS intervention and instead were seen at two timepoints, 16 weeks apart. Though the sample size is modest, a majority of participants in the current study identified as Latinx. Much intervention research is carried out with White, monolingual English-speakers. This is one of the first studies to investigate the effect of PEERS in a diverse sample in which the intervention was carried out in a language-inclusive environment in both English and Spanish, see below.

Flyers with study details were posted at community centers and events. Interested families with adolescents between the ages of 11–18 years were contacted via phone or email. Exclusionary criteria for the ASD and TD groups included: an IQ below 70, history of seizures/epilepsy, history of brain injury/disease, and a diagnosis of intellectual disability. Commonly co-occurring disorders were not exclusionary in the ASD group, though a history of serious psychiatric illness (e.g., schizophrenia, bipolar disorder) or a recent (within 6 months) psychiatric hospitalization was exclusionary. Additional exclusionary criteria for the TD group included a psychiatric diagnosis of any kind and immediate family history of ASD.

All participants in the ASD group had diagnosis confirmed with the Autism Diagnostic Observation Schedule, 2nd edition (ADOS-2) (38). The ADOS-2 was performed by research-reliable graduate students who had at least 5 years of experience working with individuals with ASD. ASD adolescents needed to have English as a primary language to be included in the intervention. Parents could speak either English or Spanish as parent groups were delivered in a bilingual format. A third timepoint set for 4 months later was scheduled to measure lasting impacts of intervention; however, COVID-19 prevented participants from returning to the lab to complete the EEG follow-up visit. This study was approved by the Institutional Review Board at the University of California, Riverside. Caregivers provided informed consent, and adolescents provided assent.

Procedures, Assessments, and Questionnaires

Cognitive abilities were tested using the 2-subtest Wechsler Abbreviated Scales of Intelligence, 2nd edition (WASI-II) (39). Composite scores were combined to create a full-scale IQ-2 (FSIQ-2). For adolescents with ASD, diagnosis was confirmed using the ADOS-2 (38). ADOS-2 consists of five modules based upon the individual's language ability and age. In this study, Modules 3 and 4 were used for participants with ASD. Willingness to participate the intervention was assessed in ASD participants using the Mental Status Checklist (21). These measures were used to confirm eligibility and therefore were not repeated.

Caregivers completed the Social Responsiveness Scale, Second Edition (SRS-2) (40), and the Social Skills Improvement System (SSIS) (41) before the intervention began (Time 1) and immediately after intervention completion (Time 2). Times 1 and 2 were ∼4 months apart, as the duration of the PEERS intervention is 16 weeks. The same EEG task was completed by adolescents in both groups at Time 1 and Time 2.

The SRS-2 is a standardized 65-item parent-report rating scale used to assess the severity of autism symptoms and social responsiveness in children ages 4 to 18 (40). A Total Score is calculated from five subscales: Social Awareness, Social Cognition, Social Communication, Social Motivation, and Restricted Interests and Repetitive Behavior.

The SSIS is a standardized 79-item parent-report measure of social and behavioral functioning for children ages 3 to 18 (41). The measure is designed to assess treatment-related changes in social skills (subscale: Social Skills) and problem behaviors (subscale: Problem Behaviors).

Teen engagement in intervention sessions was measured by tallying the number of times adolescents actively participated (e.g., asking questions, making comments, reporting on homework assignments). The tallies were recorded by the interventionist during active sessions. A sum of participation across 16 sessions was calculated. This metric is referred to below as "Teen Participation." See **Table 2** for SRS-2, SSIS, and Teen Participation means.

Social Skills Intervention: PEERS

PEERS is a 16-week, outpatient, manualized intervention to help adolescents make and keep friends (19–22, 42). The PEERS intervention consists of weekly, 1.5-h group sessions for parents and teens. Parent groups are conducted in a separate room from adolescent groups. Adolescent group sessions focused on teaching social skills specific to making and keeping friends and handling peer conflict and rejection. Skills were taught using didactic instruction which included role-play demonstrations, behavioral rehearsal activities with reinforcement and corrective feedback, and weekly homework assignments (43). Parent group sessions were provided in a bilingual format. All written parent materials were available in Spanish and English. Each group was

TABLE 2 | Mean scores on behavioral measures in TD and ASD participants at Time 1 and Time 2.

	TD		ASD	
	Time 1 M (SD)	Time 2 M (SD)	Time 1 M (SD)	Time 2 M (SD)
SRS-2 total T-score	45.29 (6.33)	44.07 (6.38)	74.85 (12.84)	68.85 (15.06)
SRS-2 social motivation T-score	49.21 (8.83)	47.43 (9.23)	75.15 (14.97)	70.77 (17.76)
SSIS social skills standard score	105.64 (11.89)	105.21 (12.59)	81.62 (19.19)	87.85 (19.05)
Teen participation	—		256.31 (91.38), range: 165–469	

Higher SRS-2 scores indicate greater severity, while lower SSIS scores indicate greater severity.

led by a trained interventionist. All procedures were supervised by a licensed psychologist.

EEG

EEG Task

The EEG task was completed by ASD and TD participants at Time 1/pre-intervention and Time 2/post-intervention. The EEG task included two blocks of 50 trials, each comprised of one of two conditions (social or non-social). In both blocks, at the beginning of each trial, a fixation cross appeared on the screen for 500 milliseconds (ms). After the fixation cross, two boxes, each containing a question mark, were displayed. Participants were instructed to indicate their guess via a button pad regarding whether the left or right stimulus was "correct." The boxes were displayed until participants made a choice—up to 3,000 ms. If participants did not make a choice after 3,000 ms the trial ended and the next trial began. After participants indicated their choice, an arrow appeared pointing in the direction of the box they picked for 3,000 ms. After 3,000 ms, feedback appeared to indicate if the participant guessed correctly or incorrectly (displayed for 1,000 ms).

In the social condition, feedback was an image of a smiling face from the "NimStim" database (44) surrounded by intact Oreo cookies for correct answers or an image of a frowning face surrounded by crossed out Oreo cookies for incorrect answers. In the non-social condition, feedback was an image of an upward arrow surrounded by Oreo cookies for correct answers or an image of a downward arrow surrounded by crossed out Oreo cookies for incorrect answers. Arrow stimuli were composed of scrambled face elements from the social condition. A computer program predetermined correct vs. incorrect answers in semi-random order such that participants got 50% "correct" and 50% "incorrect," with no more than three of the same feedback in a row. Each trial was marked to be correct vs. incorrect regardless of the participant's response.

Participants were verbally told that the reward for correct answers was Oreo cookies (or an equivalent snack). Importantly, in both the social and non-social feedback trials, the face/arrow information was incidental: it was not necessary for the participant to determine whether their response was correct. Participants were told that correct vs. incorrect responses were signaled by whether the Oreo cookies were intact or crossed out. Whether individuals viewed the social vs. non-social block first was counterbalanced. See **Figure 1**.

EEG Recording and Processing

Participants wore a standard, fitted cap (Brain Products ActiCap) with 32 silver/silver-chloride (Ag/AgCl) electrodes placed according to the extended international 10–20 system. Continuous EEG was recorded using Brain Vision Recorder with a reference electrode at Cz and re-referenced offline to average activity at left and right mastoids. Electrode resistance was kept under 50 kOhms. Continuous EEG was amplified with a directly coupled high pass filter (DC) and notch filter (60 Hz). The signal was digitized at a rate of 500 samples per second. Eye movement artifacts and blinks were monitored via horizontal electrooculogram (EOG) placed at the outer canthi of each eye and vertical EOG placed above and below the left eye.

Trials with no behavioral response, or containing electrophysiological artifacts, were excluded. Artifacts were removed via a four-step process. Data were visually inspected for drift exceeding +/−200 mV in all electrodes, high frequency noise visible in electrodes larger than 100 mV, and flatlined data. Following inspection, data were epoched and eyeblink artifacts were identified using independent component analysis (ICA). Individual components were inspected alongside epoched data, and blink components were removed. To remove additional artifacts, we utilized a moving window peak-to-peak procedure in ERPlab (45), with a 200 ms moving window, a 100 ms window step, and a 150 mV voltage threshold.

SPN

Baseline was −3,200 to −3,000 ms, and the data were epoched from −3,200 to 100 ms (time-locked to the onset of feedback stimuli). SPN mean amplitude between −210 and −10 ms was calculated for social and non-social conditions. Electrode locations included F3/F4, C3/C4, P3/P4, and T7/T8. See **Figure 2** for electrode locations.

RewP

Baseline was set to −100 to 0 ms, and the data were epoched from −100 to 800 ms. RewP mean amplitude was calculated for each condition from the frontocentral electrode, Fz (46, 47). For both conditions (face, arrow) and both feedback types (correct, incorrect), mean brain activity was calculated between 275 and 425 ms after feedback onset. The RewP was defined as a difference wave where brain activity in response to "incorrect" feedback was subtracted from brain activity in response to "correct" feedback.

N170

Upon visual inspection of grand average EEG data files, a negative-going deflection was observed after stimulus presentation, particularly in the social condition. Though the EEG stimuli in the current investigation were designed to elicit reward anticipation and response, exploratory analyses of the

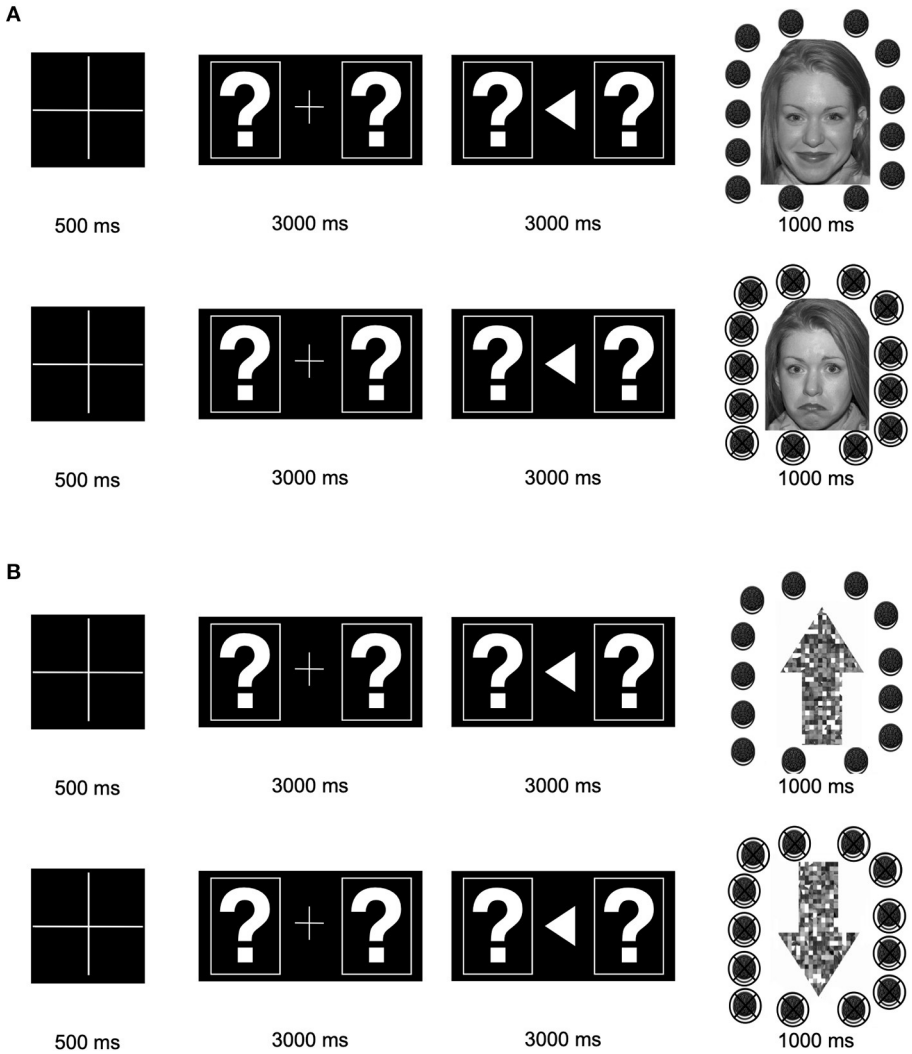

FIGURE 1 | Stimulus presentation: **(A)** Stimuli and presentation timing for the social condition. **(B)** Stimuli and presentation timing for the non-social condition. Correct feedback is shown on top (intact Oreos); incorrect feedback is shown on the bottom (crossed-out Oreos).

N170 are included. Only social and non-social trials with correct feedback (i.e., smiling faces and upwards-facing arrows) were analyzed. Incorrect trials were excluded from N170 analyses to eliminate confounds related to processing negative emotional valences (48) (i.e., frowning faces). The baseline period was set to −100 to 0 ms and data were epoched from −100 to 800 ms. Peak amplitude and latency were calculated between 150 and 250 ms in CP5/CP6 and P7/P8 electrodes (33, 49).

EEG Data Retention

Of the 13 ASD participants included in this investigation, 12 participants provided a minimum of 10 trials in the social and non-social conditions at Time 1 and Time 2. Thus, 12 ASD participants were included in analyses of the SPN, RewP, and N170.

All 14 TD were included in RewP and N170 analyses, as each participant provided a minimum of 10 trials per condition at each timepoint. For SPN analyses, four TD participants did not provide the necessary 10 trials per condition at both timepoints, resulting in a total of 10 TD participants included in SPN analyses.

Statistical Analyses

All analyses were conducted using SPSS Version 27 (2020). Repeated-measures analyses of variance (ANOVAs) were conducted to test the effects of condition (social, non-social), time (pre-, post-intervention), and group (ASD, TD) on SPN mean amplitude, RewP mean amplitude, and N170 peak amplitude and latency. ANOVAs were conducted with Age at Time 1 as a covariate.

Repeated-measures ANOVAs were conducted to test the effects of group and time on behavioral measures of interest (i.e., SRS-2, SSIS, and Teen Engagement). Pearson correlations were conducted to test which pre-intervention measures were

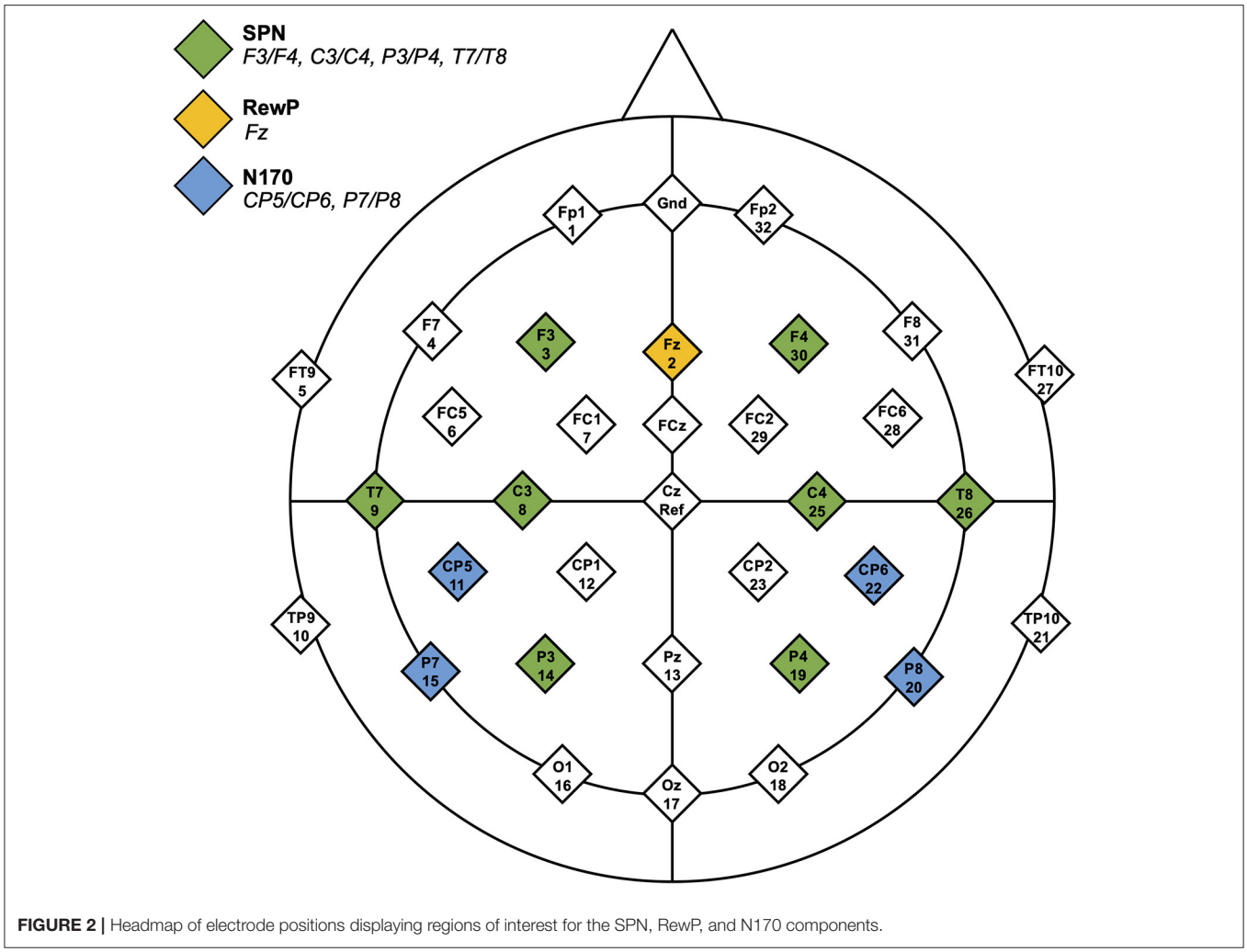

FIGURE 2 | Headmap of electrode positions displaying regions of interest for the SPN, RewP, and N170 components.

significantly associated with change in ERPs after intervention in the ASD group. Change in SPN and RewP was calculated as a difference score by subtracting pre-intervention mean amplitudes from post-intervention mean amplitudes within social and nonsocial conditions, respectively. Though there are some methodological concerns surrounding the use of change scores (e.g., reliability), they were used in this investigation due to their robustness against non-randomized designs, particularly when change scores are included as a dependent variable in regression analyses (50). Pearson correlations between behavioral variables of interest at Time 1 (pre-intervention) and ERP difference scores in the ASD group from Time 1 (pre-intervention) to Time 2 (post-intervention) were conducted to determine which variables to include in linear regression models. Finally, separate linear regressions were conducted in the ASD group based on the results of the correlations between behavioral measures at Time 1 and changes in brain activity from Time 1 to Time 2. The number of independent variables included in a multivariate regression is often determined using a 20:1 ratio, such that there should be 20 subjects for each independent variable (51, 52). Given the small sample size in this investigation, separate univariate regressions were conducted as to not violate basic principles. No prediction models including the N170 were conducted, as these analyses were exploratory.

RESULTS

ERP

SPN

Prior to running ANOVAs to test the effect of intervention and group on SPN amplitude, differences by hemisphere and electrode position were conducted using a 2 (hemisphere: left, right) × 2 (time) × 4 electrode position (Frontal, Central, Parietal, Temporal) ANOVA. No significant main effects or interactions were found. As such, ANOVAs were collapsed across hemisphere and electrode position, similar to prior investigations using the same ERP paradigm (9, 53). Note that some of these values are at the margin of statistical significance; analyses were reported for hypothesis-generating purposes and to inform future research.

A significant 2-way interaction was found between time and condition; $F_{(1,19)} = 6.07$; $p = 0.02$, $\eta_p^2 = 0.24$. A marginally

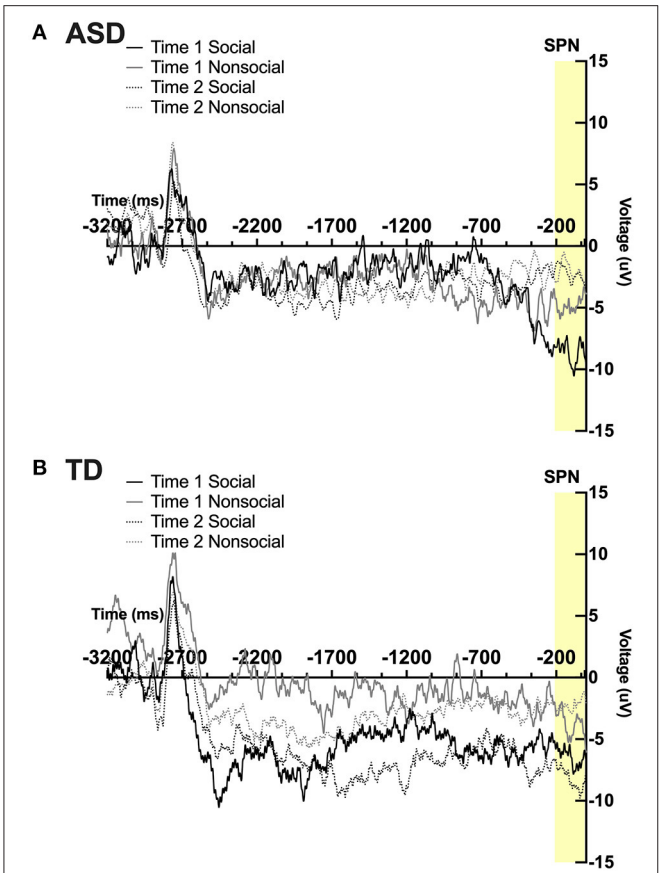

FIGURE 3 | Grand average waveforms in the social and non-social conditions at Time 1 and Time 2 from the Stimulus Preceding Negativity (SPN) in **(A)** ASD participants and **(B)** TD participants.

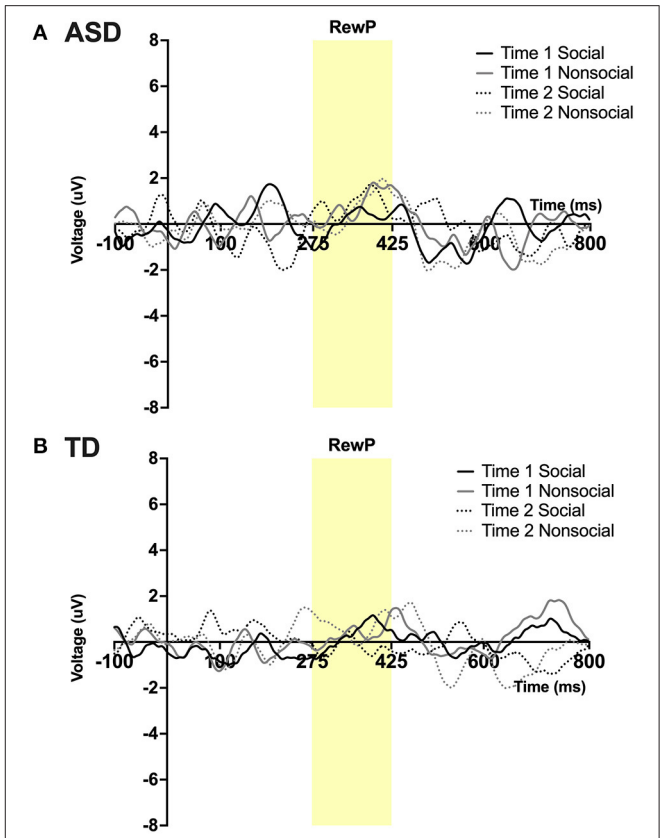

FIGURE 4 | Grand average waveforms in the social and non-social conditions at Time 1 and Time 2 from Reward Positivity (RewP) ERP in **(A)** ASD participants and **(B)** TD participants. Note that for this figure, ERPs were filtered using a 25 Hz low-pass filter.

significant 3-way interaction was found between time, condition, and group; $F_{(1,19)} = 4.09$, $p = 0.057$, $\eta_p^2 = 0.18$. Pairwise comparisons revealed a marginally significant effect of time in the ASD group, such that participants had marginally smaller SPN magnitude in the social condition at post-intervention compared to pre-intervention; $F_{(1,19)} = 4.14$, $p = 0.056$. Pairwise comparisons also revealed a marginal effect of condition at Time 2 in the TD group such that TD participants displayed a marginally more robust SPN to faces vs. arrows at time 2; $F_{(1,19)} = 3.34$, $p = 0.083$. No other main effects or interactions were observed. See **Figures 3A,B**.

RewP

A main effect of condition was found; $F_{(1,23)} = 5.15$, $p = 0.03$, $\eta_p^2 = 0.18$ such that all participants, regardless of time, had a more robust RewP mean amplitude in response to social vs. non-social stimuli. No other main effects or interactions were observed. See **Figures 4A,B**.

Exploratory Analysis: N170 Peak Amplitude

See note above; some of these values are at the margin of statistical significance. A significant 3-way interaction was found between time, hemisphere, and group; $F_{(1,23)} = 13.35$, $p = 0.045$, $\eta_p^2 = 0.16$. A 4-way interaction was found between time, condition, hemisphere, and group; $F_{(1,23)} = 14.19$, $p = 0.027$, $\eta_p^2 = 0.195$. Pairwise comparisons revealed that in the right hemisphere at Time 1, the ASD group had a more robust N170 than the TD group in the social condition; $F_{(1,23)} = 5.14$, $p = 0.033$. In the ASD group there was a marginal effect of time such that in the right hemisphere there was a more robust N170 in the social condition at Time 2 (post-intervention) compared to Time 1 (pre-intervention); $F_{(1,23)} = 3.99$, $p = 0.058$. In the TD group at Time 1, a more robust N170 was found in the non-social compared to the social condition in both left [$F_{(1,23)} = 6.08$, $p = 0.022$] and right hemispheres [$F_{(1,23)} = 4.57$, $p = 0.043$]. Additionally, a marginally significant effect of hemisphere was observed in the TD group at Time 1 in the social condition such that a more robust N170 was observed in the right vs. left hemisphere; $F_{(1,23)} = 3.86$, $p = 0.062$. See **Figure 5**.

N170 Latency

A main effect of hemisphere was observed, $F_{(1,23)} = 5.802$, $p = 0.024$, $\eta_p^2 = 0.20$, such that the left hemisphere had a shorter N170 latency than the right hemisphere. No other main effects or interactions were observed.

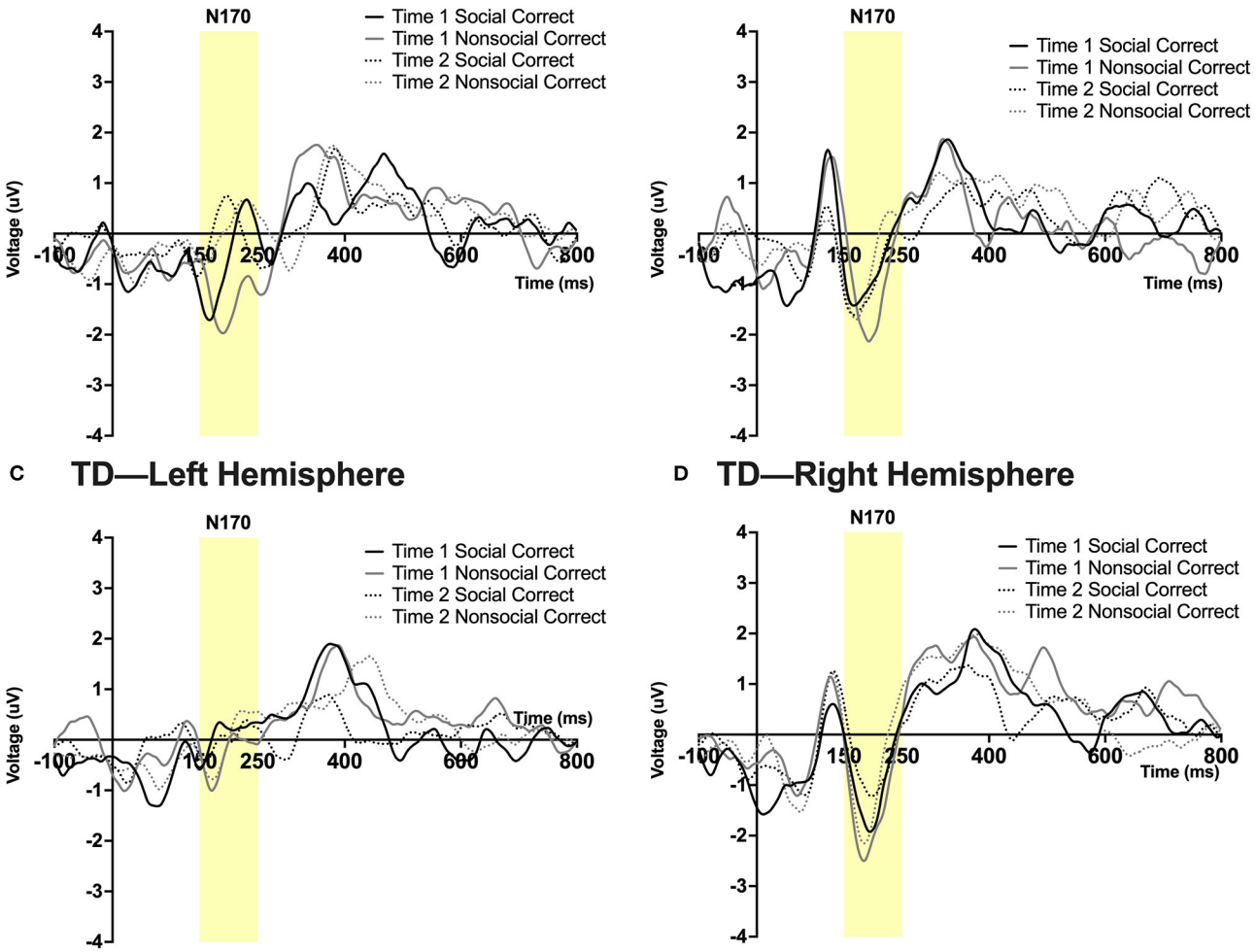

FIGURE 5 | Grand average waveforms in the social and non-social conditions at Time 1 and Time 2 for the N170 ERP in **(A)** ASD participants in the left hemisphere, **(B)** ASD participants in the right hemisphere, **(C)** TD participants in the left hemisphere, and **(D)** TD participants in the right hemisphere. Note that for this figure, ERPs were filtered using a 25 Hz low-pass filter.

Behavioral Results: Repeated Measures ANOVA

Three 2 (group) × 2 (time) repeated measures ANOVAs were conducted to measure changes in SRS-2 Total score, SRS-2 Social Motivation, and SSIS Social Skills from Time 1 to Time 2. For the SRS-2 Total score, there was a main effect of time; $F_{(1, 25)} = 9.66$, $p < 0.01$, $\eta_p^2 = 0.28$; and a significant interaction between time and group; $F_{(1, 23)} = 4.25$, $p = 0.05$, $\eta_p^2 = 0.15$. Pairwise comparisons revealed ASD participants had significantly higher SRS-2 Total scores at Time 1 [$F_{(1, 25)} = 58.94$, $p < 0.01$, $\eta_p^2 = 0.70$] and Time 2 [$F_{(1, 25)} = 31.84$, $p < 0.01$, $\eta_p^2 = 0.56$] compared to TD participants. ASD SRS-2 Total scores decreased from Time 1 to Time 2; $F_{(1, 25)} = 12.88$, $p < 0.01$, $\eta_p^2 = 0.34$, while TD scores remained the same across time, $F_{(1,25)} = 0.59$, $p = 0.49$. A main effect of group was observed for the SRS-2 Social Motivation subscale [$F_{(1, 25)} = 27.26$, $p < 0.001$, $\eta_p^2 = 0.52$] and SSIS Social Skills subscale, [$F_{(1, 25)} = 12.88$, $p < 0.01$, $\eta_p^2 = 0.34$], such that TDs had lower Social Motivation T-scores and higher Social Skills Standard Scores than ASD participants, regardless of time. Note that for the SRS-2, lower scores indicate fewer symptoms of ASD, whereas on the SSIS, higher sores indicate fewer social skills impairments. Refer to **Table 2** for mean values.

ERP and Behavior: Correlations and Linear Regressions

Correlations

Note that some of these values are at the margin of statistical significance. The SPN social condition mean amplitude change was marginally correlated with pre-intervention age ($r = -0.56$, $p = 0.059$) and pre-intervention SRS-2 Social Motivation scores ($r = -0.57$, $p = 0.055$). Thus, increased magnitude of the SPN from Time 1 to Time 2 (note that the SPN more negative change

TABLE 3 | Results of correlations and linear regressions in the ASD Group only.

	Correlation		Linear regression				
	r	*p*	*B*	SE B	*β*	*t*	*p*
SPN social condition change							
Age T 1	−0.56	0.059	−3.27	1.53	−0.56	−2.133	0.059
SRS-2 social motivation T 1	−0.57	0.055	−0.484	0.22	−0.57	−2.17	0.055
SRS-2 total T 1	−0.53	0.079	–	–	–	–	–
SSIS social skills T 1	0.54	0.069	–	–	–	–	–
RewP social condition change							
SRS-2 social motivation T 1	−0.67	0.02	−0.32	0.11	−0.67	−2.85	0.02
Teen participation	0.70	0.01	0.05	0.02	0.70	3.10	0.01

SPN social condition change and RewP social condition change are each outcome variables; all regressions were run separately.

scores reflect more robust reward anticipation) was correlated with older ages and worse social motivation prior to the start of intervention. Two additional correlations with the SPN social condition mean amplitude change trended toward significance. SPN mean amplitude change was negatively correlated with SRS-2 Total ($r = -0.53$, $p = 0.079$) and positively correlated with SSIS Social Skills ($r = 0.54$, $p = 0.069$).

The RewP social condition mean amplitude change was negatively correlated with SRS-2 Social Motivation scores pre-intervention ($r = -0.67$, $p = 0.02$), such that an increased reward response to social stimuli was correlated with better social motivation scores before the start of intervention. RewP social condition difference score was positively correlated with Teen Participation ($r = 0.70$, $p = 0.01$), such that increased reward response to social stimuli from Time 1 to Time 2 was correlated with more intervention engagement. See **Table 3** for a summary of correlation and linear regression results.

Linear Regressions

As stated above, some of these values are at the margin of statistical significance. Two linear regressions were conducted to test if age at the start of intervention and pre-intervention SRS-2 Social Motivation scores predicted change in SPN social condition mean amplitude. Thirty-two percent of the variance of the change in anticipation of social reward was accounted for by SRS-2 Social Motivation pre-intervention scores, $\beta = -0.57$; $F_{(1,10)} = 4.71$, $p = 0.055$. Thirty-one percent of the variance in change in anticipation of social reward was accounted for by age at the start of intervention, $\beta = -0.56$; $F_{(1,10)} = 4.55$, $p = 0.059$.

Two linear regressions were conducted in the ASD group to test if pre-intervention SRS-2 Social Motivation scores and Teen Participation predicted change in RewP social condition mean amplitude. Results revealed that 44.9% of the variance of the change in social reward responsivity (RewP mean amplitude in response to faces) was accounted for by SRS-2 Social Motivation pre-intervention scores, $\beta = -0.67$; $F_{(1,10)} = 8.14$, $p = 0.02$. Similarly, 49% of the variance of the change in social reward responsivity was accounted for by Teen Participation, $\beta = 0.70$; $F_{(1,10)} = 9.60$, $p = 0.01$.

DISCUSSION

Social behaviors were improved in adolescents with ASD in the areas of social responsiveness and social skills, such that a reduction in autism symptomatology was observed after participation in PEERS. In addition to behavioral improvements, changes in neural correlates of reward were detected. The primary aim of this study was to investigate anticipation of and response to reward-related brain activity before and after completion of PEERS and to examine the ways in which individual factors impacted outcomes. As such, this preliminary study is one of the first to examine reward-related brain activity before and after intervention with a group of teens with ASD. Additionally, this investigation included a majority Latinx sample, a historically underrepresented group. The inclusion of minority groups in intervention and in measures of neural response advances the representation of such groups and improves generalizability of findings.

Anticipation

Participants with ASD displayed marginally less anticipation (less robust SPN) to social rewards at post-intervention compared to pre-intervention. Though contrary to our hypotheses, it is possible that increased comfort and familiarity with social situations may explain these findings. That is, increased familiarity and experiences in social settings and/or in social interactions may have dampened anticipation of social information, as social behaviors became routine throughout the course of intervention. In contrast, TD participants did not evidence differences in reward anticipation across time. However, marginal differences between social and non-social conditions were observed at Time 2 such that TD adolescents evidenced more anticipatory brain activity in response to social vs. non-social stimuli. Our findings suggest that participation in PEERS leads to changes in anticipation of social stimuli for adolescents with ASD, whereas time does not lead to equivalent changes for TD adolescents.

Individual variability of change in neural correlates of social anticipation from pre- to post-intervention was predicted by

age and parent-reported social motivation at the beginning of the intervention. Older adolescents and those with *less* reported social motivation prior to PEERS displayed increased neural anticipation for faces from pre- to post-intervention. It will be important for future research to explore potential effects of age on PEERS efficacy, as the intervention is inclusive of a large age range. Our finding that teens with less social motivation prior to PEERS displayed increased social reward anticipation after PEERS is a critical step forward in our ability to understand why some participants may benefit more from intervention than others.

Processing

In all participants, response to rewards was greater (more robust RewP) to social compared to non-social stimuli. Though previous work has reported hypoactivation in reward-related brain areas to social stimuli (54), findings in the current study provide an alternative account. It is possible that social deficits unique to ASD may not be reliably detected at the neural level in all children/adolescents, indicating that behavioral and objective measures of social response may not always be aligned. This is an important consideration when using objective measures of neural activity and emphasizes the need to examine individual variables in addition to group differences. It is important to keep in mind that one of the criteria for participation in PEERS is that teens with ASD be motivated to make and keep friends; as such, teens in the current study were distinctly socially motivated. Consequently, future studies measuring neural changes before and after intervention in adolescents and/or adults with ASD should consider participant motivation, as it is often required in these groups.

Although between-group differences were not observed, within-group variability of adolescents with ASD shed light on individual differences that affect social reward responsivity after intervention. Individual change in neural correlates of response to social reward was predicted by parent-reported social motivation before intervention and active engagement during the program. Participants who were *more* actively engaged in PEERS and who displayed *more* social motivation prior to the start of intervention made the biggest gains in neural response to social rewards from pre- to post-intervention. Findings related to teen participation during intervention underscore the importance of engagement during behavioral intervention.

The effect of parent-reported social motivation prior to PEERS on changes in brain activity related to reward processing is the opposite of what we observed for social reward anticipation. That is, adolescents who had *lower* levels of parent-reported social motivation prior to PEERS displayed *greater* increases in neural correlates of social anticipation after PEERS, yet adolescents who had *higher* levels of parent-reported social motivation before PEERS displayed *increased* neural correlates of social reward responsivity after PEERS. This underscores the importance of dissociating social reward anticipation from social reward processing when considering individual response to intervention, as these constructs likely represent different neural processes. It may be that there are differences in saliency between wanting/anticipating social rewards vs. liking/responding to social rewards (55, 56) within the brain's reward system in individuals with ASD. These distinct cognitive processes offer a unique understanding of the Social Motivation Theory in adolescents with ASD who are driven to make and keep friends, suggesting that both motivation and reward systems may moderate intervention effects.

Exploratory N170 Findings

Exploratory analyses were performed on the N170. A more robust N170 response approached significance at post-intervention compared to pre-intervention in the ASD group within the right hemisphere. This indicates an enhancement of facial processing after intervention that mirrors findings in neurotypical populations (32). It is important to note that the stimuli and ERP paradigm used in the current investigation were not designed to elicit N170 responses and thus differ from traditional measurements of the N170 (e.g., facial stimuli were positive in valence and contained additional reward-related information). Thus, findings from the N170 should be interpreted with caution.

Limitations

Some limitations must be considered when interpreting results. Our sample size is small, and thus may have been underpowered to detect between-group differences. Inclusion of an ASD wait-list control group would have improved the experimental design of the investigation and may have allowed for the effects of the "natural passage of time" vs. "intervention" to be disentangled in the ASD group. However, inclusion of a TD group established, in-part, that change was not solely due to the passage of time. Change scores were used in this investigation instead of alternative methods of pre- and post-test analyses, which may have influenced results. A clustered design was not utilized in this design and this may have impacted our statistical power and effect size of intervention effects (57). Additionally, a small sample size reduces our ability to generalize our findings to larger groups of adolescents with ASD. Given the cognitive demands of PEERS and the EEG procedures, participants were required to have cognitive abilities in the average range to be eligible for the current study (i.e., IQ $\leq$ 70). Another requirement was for teens with ASD to be motivated to make and keep friends and for both parents and teens to be able to attend weekly 90-min intervention sessions for 16 weeks. Given these considerations, it is likely that participants in the current study represent a subset of adolescents with ASD. In the future, it will be important to clarify which of these factors may affect the efficacy of PEERS.

Conclusion

To our knowledge, this is the first study to measure neural correlates of both social reward anticipation and processing in adolescents with ASD before and after the PEERS intervention. Findings supported our hypothesis that change in neural correlates of social reward anticipation and processing can be predicted by individual characteristics prior to intervention. Although traditional conceptualizations of social motivation define this construct as the desire or intention to engage and interact with others, our findings reinforce previous work

that reward anticipation and reward processing are dissociable constructs (56, 58). Our findings suggest that for individuals with ASD who may have lower levels of intrinsic motivation to interact with others, PEERS may enhance their desire to approach others, commonly known as approach motivation, or "wanting" to interact (as indicated by increased neural reward anticipation to faces; SPN). However, for those who are already motivated to interact with others, completion of the PEERS program may further reinforce social interactions as pleasant (as indicated by increased neural reward processing of faces, RewP).

In ASD intervention research, there remains a lack of validated biomarkers that can be used to predict intervention outcomes (59). Future studies with larger samples should attempt to both replicate these findings and further parse these constructs to move closer to "precision medicine" efforts to individualize intervention and predict which adolescents are most likely to benefit from PEERS.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Board at the University of California, Riverside. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

KS designed the experiment. EB and KS conceptualized the analysis strategy. EB performed the EEG processing, statistical analysis, statistical interpretation, and drafting of the manuscript under the supervision of KS. JB verified the analytical methods and interpretations. EV and TC reviewed and confirmed descriptions of methodology. All authors discussed the results, contributed to the final published manuscript, and have read and agreed to the published version of the manuscript.

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Saliva RNA Biomarkers of Gastrointestinal Dysfunction in Children With Autism and Neurodevelopmental Disorders: Potential Implications for Precision Medicine

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Gastrointestinal (GI) disorders are common in children with neurodevelopmental disorders such as autism spectrum disorder (ASD). A limited understanding of the biologic factors that predispose this population to GI disorders has prevented development of individualized therapies to address this important medical issue. The goal of the current study was to determine if elements of the salivary micro-transcriptome could provide insight into the biologic perturbations unique to children with ASD-related GI disturbance. This cohort study included 898 children (ages 18–73 months) with ASD, non-ASD developmental delay (DD), or typical development (TD). The saliva micro-transcriptome of each child was assessed with RNA-seq. Outputs were aligned to microbial and human databases. A Kruskal Wallis analysis of variance (ANOVA) was used to compare levels of 1821 micro-transcriptome features across neurodevelopmental status (ASD, DD, or TD) and GI presence or absence. An ANOVA was also used to compare micro-transcriptome levels among GI sub-groups (constipation, reflux, food intolerance, other GI condition, no GI condition), and to identify RNAs that differed among children taking three common GI medications (probiotics, reflux medication, or laxatives). Relationships between features identified in ANOVA testing were examined for associations with scores on the Autism Diagnostic Observation Schedule, 2nd Edition (ADOS-2) and the Vineland Adaptive Behavior Scales. GI disturbance rates were higher among children with ASD than peers with TD but were similar to those with DD. Five piwi-interacting RNAs and three microbial RNAs displayed an interaction between developmental status and GI disturbance. Fifty-seven salivary RNAs differed between GI sub-groups–with microRNA differences between food intolerance and reflux groups being most common. Twelve microRNAs displayed an effect of

GI disturbance and showed association with GI medication uses and measures of behavior. These 12 microRNAs displayed enrichment for 13 physiologic pathways, including metabolism/digestion long-term depression, and neurobiology of addiction. This study identifies salivary micro-transcriptome features with differential expression among children with ASD-related GI disturbance. A subset of the RNAs displays relationships with treatment modality and are associated with autistic behaviors. The pathobiologic targets of the micro-transcriptome markers may serve as targets for individualized therapeutic interventions aimed at easing pain and behavioral difficulties seen in ASD-related GI disturbance.

Keywords: biomarkers, saliva, RNA, microRNA, autism (ASD), gastrointestinal

INTRODUCTION

Previous work has demonstrated that the salivary micro-transcriptome (non-coding RNA and microbial RNA) could be used to distinguish between children with autism spectrum disorder (ASD) (ages 2–6 years) and peers with typical development or developmental delay (1). These non-coding RNAs have regulatory roles in metabolism, cell differentiation and neuronal differentiation, by inhibiting gene expression (2). Elements of the micro-transcriptomes are up- or down-regulated by cells in response to the external environment (3), which suggests that they may have a dynamic relationship with other factors. Several non-coding RNA in saliva have demonstrated that their levels are associated with adaptive and autistic behaviors in children with ASD (4, 5), and are associated with socialization and autistic behaviors in young children with ASD (5). However, the relationship between non-coding RNA and comorbid conditions in ASD is not yet known.

Children with ASD appear to more frequently experience GI conditions than their neurotypical peers. Children with ASD have been reported to be diagnosed with a GI problem almost four times more often than children without ASD (6). The range of reported prevalence of GI symptoms is from 9 to 91% (7), likely a result of different methods of GI assessment. Constipation and diarrhea tend to be the most common GI diagnoses in ASD (6), with constipation the most common (8). Constipation can frequently be sufficiently severe to result in emergency department visits and hospital admissions among children with ASD (9).

Children with abdominal pain can also manifest difficult and distressing behaviors such as irritability, social withdrawal, stereotypy, and hyperactivity, as well as aggression and self-injurious behaviors (7, 10, 11). Associated comorbid conditions can include seizures, anxiety, depressed mood, attention-deficit/hyperactivity disorder, oppositional defiant disorder, sleep problems, as well as other problem behaviors (12–16). Problem behavior may, itself, sometimes be an indicator of GI distress in ASD, particularly among individuals with ASD with limited language (7). Younger individuals with ASD and GI disturbances display more externalizing behaviors such as aggression, and older individuals with ASD display more internalizing symptoms such as anxiety and depression (16). Stress reactivity as well as anxiety and autonomic arousal are also critically interrelated to severity of GI symptoms in ASD (17, 18). Many ASD patients with GI disturbances, such as constipation, are less likely to respond to first line therapies, such as stool softeners (19). Therefore, a better understanding of the biologic processes driving GI disturbances in patients with ASD might provide mechanistic insights toward better treatments for GI pain and related behaviors.

Heterogeneity across the autism spectrum has led to failures in many of the early clinical trials attempting to target core features of ASD (20). While micro-transcriptomes appear to distinguish ASD patients from neurotypical controls (21), they may also have particular value in helping to distinguish specific subtypes of ASD, which might impact treatment. Because of the significant adverse effects of GI disorders in ASD (6, 9), as well as their interrelationships with behavioral disturbances (12–16), we sought to examine differential micro-transcriptome expression in ASD patients with and without GI disturbances, as well as how this interrelates with behaviors. A greater understanding of the downstream targets of the differentially expressed micro-transcriptomes may help guide future personalized medicine approaches to treatment of GI disturbances in ASD.

METHODS

Ethics

The study was approved by the Western Institutional Review Board (IRB #20180172). Written consent was obtained for all participants, and written informed assent was documented for those capable of assent.

Participants

This case control study included a total of 898 children, ages 18–73 months, who were recruited from outpatient pediatric clinics affiliated with seven academic medical centers: Penn State University ($n = 312$), State University of New York (SUNY) Upstate Medical University ($n = 335$), Missouri University ($n = 108$), Cincinnati Children's Hospital ($n = 45$), Texas Children's Hospital ($n = 54$), University of California Irvine ($n = 15$); and University of Iowa ($n = 29$). Participants were divided into three groups based on neurodevelopmental status: autism spectrum disorder (ASD; $n = 503$), non-ASD developmental delay (DD; $n = 205$), and typical development (TD; $n = 190$). ASD status was determined by trained clinicians using

DSM-5 criteria in association with standardized assessment tools (i.e., the Autism Diagnostic Observation Schedule, 2nd Edition; ADOS-2). DD participants included children referred for initial ASD assessment who did not meet diagnostic criteria, as well as children with negative ASD screening tools who required early intervention services for delays in gross motor, fine motor, language, or cognitive development (as reported by parental survey and confirmed through review of the medical record). TD participants included children recruited at the time of their annual well child visits who did not exhibit developmental delays on standard developmental surveillance tools (e.g., Survey of Wellbeing in Young Children, Parents' Evaluation of Developmental Status–Developmental Milestones, Modified Checklist for Autism in Toddlers Revised). Participants were further subdivided by presence ($n = 184$) or absence ($n = 714$) of gastrointestinal (GI) disturbance, based on: (2) parent report of (a) constipation ($n = 84$); (b) reflux ($n = 46$); (c) chronic diarrhea or abdominal pain ($n = 22$); (d) food intolerance ($n = 45$); (e) cyclic vomiting/dysphagia ($n = 3$); or (f) eosinophilic esophagitis ($n = 7$). Note that total numbers of specific conditions exceed the number of participants with a GI disturbance because 23 participants reported more than one type of GI disturbance. Exclusionary criteria for all participants included Ward of the State, g-tube dependence, active periodontal infection or acute upper respiratory illness.

Measures

Participant Characteristics

The ADOS-2 was administered by trained raters to children with ASD ($n = 409$), and children with DD ($n = 121$) in whom ASD was suspected. The Vineland Adaptive Behavioral Scales 3rd Edition (VABS-III) was used to measure adaptive behavior, communication, and social interaction for all participants. Additionally, medical and demographic information including sex, age, race, ethnicity, medical conditions, and medications, was collected through parental surveys and affirmed via review of the electronic health record where available.

Saliva Collection and Processing

Saliva was obtained from all participants in a non-fasting state via swab, targeting the base of the tongue and between the gums and buccal mucosa as locations for the collection using an Oracollect RNA swab (DNA Genotek, Ottawa, Canada). Nucleic acid extraction was performed using the Qiagen miRNeasy Microkit (Cat. No. 217084), a QIAzol based purification method. The RNA sequencing process included using an Illumina TrueSeq Small RNA Prep protocol for library construction, followed by sequencing on an Illumina flow-cell and a NextSeq 500 instrument (Illumina; San Diego, CA, United States). Sequencing outputs were a binary base call (BCL) sequence file per sample, which was then converted to a FASTQ file, a text-based format that includes detected bases and associated quality scores (i.e., confidence in correct detection). Alignment and quantification of known RNA sequences for each collected specimen was done using the Bowtie1 aligner (22) to the following reference databases: miRBase v22 (23), piRBase v1 (24), RefSeq v90 (25), and hg38. Quantification of the detected sequences yielded counts of known human micro-ribonucleic acids (miRNAs), long non-coding transcripts (small nucleolar RNAs), and piwi-interacting RNA (piRNAs). To determine microbial RNAs present in the sample, the leftover sequences that did not align to hg38 were aligned to the NCBI microbial database using k-SLAM, an efficient aligner used in metagenomic data. Aligned sequences were then assigned to microbial genes, which were quantified to a microbial identity (e.g., genus, species, strain). Prior to analysis and count normalization, low count RNAs were removed from further analysis so that only reliably expressed RNAs were interrogated. Tabulated counts of each RNA were compared to the total counts detected in that RNA category, and RNAs that did not account for at least 0.01% of the total were dropped. Following abundance filtering, the remaining RNAs were quantile normalized and mean-center scaled.

Statistics

The primary goals of the study were to: (2) identify human and microbial RNA levels in saliva that were associated with GI disturbance; (3) investigate whether these relationships were impacted by child developmental status; and (4) determine if specific RNA "biomarkers" displayed unique expression patterns in particular GI disturbances (e.g., constipation) or with particular treatments (e.g., probiotics). A two-way analysis of variance (ANOVA) was used to compare levels of 1821 RNA among the 898 participants based upon two factors: (2) neurodevelopmental status (ASD, DD, or TD); and (3) GI status (presence or absence of any GI condition). Interactions between neurodevelopmental status and GI status were reported. A one-way Kruskal Wallis rank sum test was used to identify RNAs that differed among GI sub-groups (constipation, reflux, food intolerance, other GI condition, no GI condition), and to identify RNAs that differed among those taking three common GI medications (probiotics, reflux medication, or laxatives). Finally, given the potential associations between underlying GI disturbance and child behaviors, relationships between RNAs identified in ANOVA testing, as well as the all one-way Kruskal Wallis testing were examined for associations with scores on the ADOS-2 and Vineland using Spearman Rank Testing. Benjamini Hochberg multiple testing correction was applied to all analyses. Functional analysis of candidate miRNAs (features displaying an interaction effect between neurodevelopmental status and GI disturbance, as well as relationships with treatment or behavior) was performed in DIANA miRPATH software v3.0 (26). The microT-cds algorithm (0.95 microT Threshold) was used to identify pathways over-represented by putative messenger RNA targets by Fisher Exact Test with Benjamini Hochberg multiple testing correction. Additionally, the rates of different demographic features were tested in the population. To test for differences in age by diagnosis (ASD, DD, TD) or presence of a GI disturbance, a one-way ANOVA was used. To test for differences in rates of sex, race, ethnicity, GI disturbance, constipation, reflux, food intolerance, chronic abdominal pain, diarrhea, or eosinophilic esophagitis, a chi-squared test was used yielding the chi-squared test statistic (x) and the associated p-value (p).

TABLE 1 | Participant characteristics.

	All ($n = 898$)	ASD ($n = 503$)	DD ($n = 205$)	TD ($n = 190$)
Age, months (SD)	44 (16)	44 (16)	43 (15)	47 (18)
Sex, # male (%)	663 (73)	399 (79)	147 (71)	117 (61)*
White, # (%)	691 (76)	373 (74)	160 (78)	158 (83)*
Black, # (%)	115 (12)	72 (14)	29 (14)	14 (7)*
Asian, # (%)	22 (2)	17 (3)	3 (1)	2 (1)
Other race, # (%)	100 (11)	55 (10)	25 (12)	20 (10)
Hispanic, # (%)	99 (11)	70 (13)	18 (8)	11 (5)*
GI disturbance, # (%)	184 (20)	114 (22)	50 (24)	20 (10)*
Constipation, # (%)	84 (9)	57 (11)	20 (9)	7 (3)*
Reflux, # (%)	46 (5)	35 (6)	10 (4)	1 (0.5)*
Food intolerance, # (%)	45 (5)	23 (4)	11 (5)	11 (5)
Chronic abdominal pain or diarrhea, # (%)	22 (2)	16 (3)	6 (2)	1 (0.5)
Cyclic vomiting or dysphagia, # (%)	3 (0.3)	0 (0)	3 (1)*	0 (0)
Eosinophilic esophagitis, # (%)	7 (0.7)	4 (7)	3 (1)	0 (0)

*The number of participants with specific GI disturbances exceeds the total number of participants with any GI disturbance (n = 184) because 22 participants reported more than one GI disturbance. *Denotes significant difference ($p < 0.05$) compared with ASD group on chi-square testing.*

RESULTS

Participants

Participating children had an average age of 44 (±16) months. They were mostly Caucasian (691/898, 76%), non-Hispanic (799/898, 89%), and male (663/898, 73%) (**Table 1**).

There were more males in the children with ASD (399/503, 79%) than in the children with TD (117/190, 61%) ($p = 0.00000177$, $x = 22.83$). There were fewer children with ASD who reported White race (373/503, 74%) than children with TD (158/190, 83%) ($p = 0.0125$, $x = 6.24$). More children with ASD reported Black race (72/503, 14%) and Hispanic ethnicity (70/503, 13%), compared to children with TD (14/190, 7%; 11/190, 5%, respectively) ($p = 0.0134$, $x = 6.12$; $p = 0.00297$, $x = 8.82$, respectively). There was no difference between ASD/DD/TD groups in age ($p = 0.0588$). There were limited differences between ASD/DD groups in sex ($p = 0.029$, $x = 4.79$), and no differences in reported White race ($p = 0.276$, $x = 1.19$), Black race ($p = 0.954$, $x = 0.00335$), or ethnicity ($p = 0.0603$, $x = 3.53$).

A higher proportion of children with ASD reported GI disturbance (114/503, 22%) than children with TD (20/190, 10%) ($p = 0.000307$, $x = 13.03$). Among children with ASD, reported rates of constipation (57/503, 11%) and reflux (35/503, 6%) were higher than reported rates among children with TD (7/190, 3%; and 1/190, 0.5%, respectively) ($p = 0.00416$, $x = 8.21$; $p = 0.0025$, $x = 9.14$, respectively). There were no differences between children with ASD and children with DD in rates of constipation ($p = 0.78$, $x = 0.077$), reflux ($p = 0.46$, $x = 0.54$), food intolerance ($p = 0.86$, $x = 0.031$), chronic abdominal pain ($p = 0.77$, $x = 0.085$), diarrhea ($p = 0.797$, $x = 0.066$), or eosinophilic esophagitis ($p = 0.415$, $x = 0.664$). There was no difference in age ($p = 0.205$), sex ($p = 0.87$, $x = 0.0255$), White race ($p = 0.909$, $x = 0.0132$), Black race ($p = 0.395$, $x = 0.723$), and limited differences in ethnicity ($p = 0.041$, $x = 4.15$) between children with/without GI disturbance.

Impact of GI Disturbance on Saliva RNAs

Among the 1821 RNA features interrogated, 28 displayed a significant difference (adj $p < 0.05$) between children with/without GI disturbance (**Table 2A**). These RNA features included four mature miRNAs and 24 small non-coding RNAs, but no microbial RNAs. There were eight RNA features that displayed a significant interaction effect between neurodevelopmental status (ASD/DD/TD) and presence/absence of GI condition (**Table 2B**). These RNA features included five piRNAs and three microbial RNAs (**Figure 1**). The piRNAs tended to display similar saliva levels across ASD/DD/TD groups without GI disturbance, but were lower among children with ASD and GI disturbance, relative to peers with TD and GI disturbance.

Differences in Saliva RNA Levels Among GI Phenotypes

There were 57 RNA features that differed between GI phenotypes (**Table 3**). These RNA features included 12 microbial RNAs, three piRNAs, and 42 miRNAs. The largest differences tended to occur in miRNA levels, and were most common between children with reflux and food intolerance (**Figure 2**).

Effect of Medications on Saliva RNAs

Levels of 65 RNA features differed among children with GI disturbance on probiotics ($n = 22$) and children with GI disturbance not taking probiotics ($n = 162$) (**Supplementary Table 1**). These RNA features included 37 miRNAs, 75 piRNAs, one small non-coding RNA, and one microbial RNA. Levels of 53 RNA features differed among children with GI disturbance on laxatives (i.e., polyethylene glycol; $n = 39$) and children with GI disturbance not taking laxatives ($n = 145$). These RNA features included 15 microbial RNAs, seven small non-coding RNAs, four piRNAs, 27 miRNAs.

TABLE 2A | Transcripts with significant differences between children with and without GI disturbances.

Transcript	*P*-value	Critical value
hsa-miR-224-5p	0.000493513	3.52E-04
hsa-miR-27a-3p	0.000602833	7.04E-04
hsa-miR-27b-3p	0.000609137	0.001056338
hsa-miR-151a-5p	0.001025943	0.001408451
NR_029493.1	0.000202054	3.79E-04
NR_002579.1	0.001114037	7.58E-04
NR_000007.1	0.0019312	0.001136364
NR_003689.1	0.001943995	0.001515152
NR_145802.2	0.002630256	0.001893939
NR_002439.1	0.002949461	0.002272727
NR_003688.1	0.003909225	0.002651515
NR_002744.1	0.005420832	0.003030303
NR_023363.1	0.005674379	0.003409091
NR_023364.1	0.005674379	0.003787879
NR_023365.1	0.005674379	0.004166667
NR_023366.1	0.005674379	0.004545455
NR_023367.1	0.005674379	0.004924242
NR_023368.1	0.005674379	0.00530303
NR_023369.1	0.005674379	0.005681818
NR_023370.1	0.005674379	0.006060606
NR_023372.1	0.005674379	0.006439394
NR_023373.1	0.005674379	0.006818182
NR_023374.1	0.005674379	0.00719697
NR_023375.1	0.005674379	0.007575758
NR_023376.1	0.005674379	0.007954545
NR_023377.1	0.005674379	0.008333333
NR_023378.1	0.005674379	0.008712121
NR_023379.1	0.005674379	0.009090909

TABLE 2B | Transcripts displaying a significant interaction effect between neurodevelopmental status and the presence/absence of GI condition.

Transcript name	*P*-value	Critical value
piR-hsa-6148	0.000493282	2.89E-04
piR-hsa-6145	0.000565113	5.78E-04
piR-hsa-6147	0.000566969	8.67E-04
piR-hsa-6146	0.000571064	0.001156069
piR-hsa-6144	0.000591411	0.001445087
Jeotgalibaca	3.31E-05	3.70E-04
Methylophilus sp. TWE2	0.000209947	2.20E-04
Jeotgalibaca sp. PTS2502	0.000398509	4.41E-04

Relationship of GI-Related Saliva RNAs and Child Behavior

There were 224 RNA features that displayed a significant relationship (adj $p < 0.05$) with at least one measure of child behavior on the VABS or the ADOS-2 (**Supplementary Table 2**). These RNA features included 47 miRNAs, 69 piRNAs, 16 small non-coding RNAs, and 92 microbial RNAs. The largest number of relationships were observed between RNA features and Vineland Communication Scores ($n = 132$).

Functional Implications of Saliva miRNA Candidates

There were 12 salivary miRNAs that displayed relationships with GI disturbance, GI medications, and child behavior (miR-1307-5p, miR-141-3p, miR-142-5p, miR-148a-5p, miR-186-5p, miR-200a-3p, miR-200a-5p, miR-23a-3p, miR-23b-3p, miR-28-3p, miR-532-5p, and miR-769-5p). Together, these 12 miRNAs display enrichment for 13 KEGG pathways, including several implicated in metabolism/digestion (steroid biosynthesis, porphyrin metabolism, drug metabolism, ascorbate metabolism, lysine degradation, calcium reabsorption, and thyroid hormone signaling), and neurobiology (long-term depression, morphine addiction) (**Table 4**).

DISCUSSION

In this cohort study of 898 children, rates of GI disturbance were higher among children with ASD than peers with TD, as expected (6), but were similar to those with DD. There were five piRNAs and three microbial RNAs in saliva that displayed an interaction between developmental status and GI disturbance (**Figure 1**). These features may serve as biomarkers for the unique pathophysiology leading to elevated GI disturbance in children with ASD. There were many salivary RNAs whose levels differed between GI disturbance phenotypes–with miRNA differences between food intolerance and reflux groups being most common. Levels of 12 salivary miRNAs that displayed an effect of GI disturbance were also associated with GI medications and measures of child behavior (miR-1307-5p, miR-141-3p, miR-142-5p, miR-148a-5p, miR-186-5p, miR-200a-3p, miR-200a-5p, miR-23a-3p, miR-23b-3p, miR-28-3p, miR-532-5p, and miR-769-5p).

The 12 salivary miRNAs that displayed relationships with GI disturbance, GI medications, and child behavior may serve as examples of biologic targets for personalized diagnostic and therapeutic approaches in children with ASD-related GI disturbance. Putative targets of these 12 miRNAs include transcripts that code for key regulators of both metabolism (e.g., steroid biosynthesis, porphyrin metabolism, ascorbate metabolism, calcium reabsorption, thyroid hormone signaling) and neurobiology (e.g., long-term depression). Intriguingly, exogenous steroids, porphyria, hypercalcemia, hypothyroidism, and depression are all associated with constipation and/or abdominal pain. It is possible that the 12 miRNAs contribute to sub-clinical perturbations in these physiologic pathways, in so-much-as they lead to GI pain without causing other overt clinical symptoms. For example, rodent models have demonstrated that restoration of miR-148a expression in the lower GI tract may reduce colitis (27), while elevations in miR-200a may lead to irritable bowel-like symptoms through inhibition of serotonin and cannabinoid transporters (28).

Our understanding of the nature of GI disturbances in ASD is only beginning to emerge. Numerous pathways, including

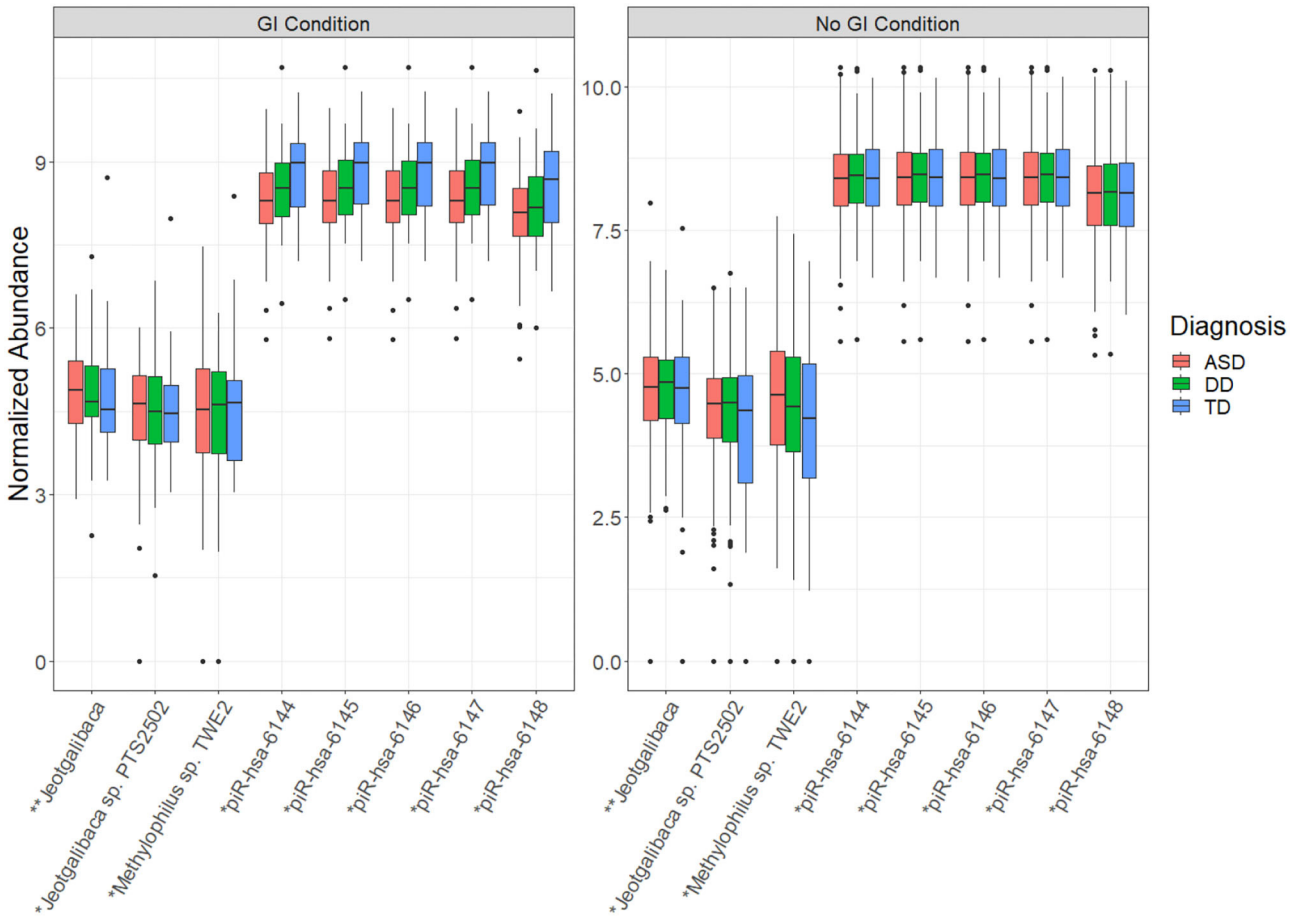

FIGURE 1 | Transcript abundance displaying significant interaction between neurodevelopmental status and presence/absence of GI condition. The whisker box plots represent normalized abundance of microbial RNAs and piRNAs that displayed a significant interaction between neurodevelopmental status (ASD/DD/TD) and presence/absence of any GI condition. These features may provide insight into the unique biology underlying the heightened prevalence of GI conditions in children with ASD. Significance levels: p-val < 0.001 (*), p-val < 0.0001 (**). For all features shown, the adjusted p-value (FDR) <0.05.

autonomic arousal (17, 18), serotonin dysregulation (29), and perturbations in gene expression (30) have all been implicated in this process. The micro-transcriptome features identified in this study provide a single mechanism through which each of these pathways may converge (**Figure 3**). For example, recent research has found that stress reactivity, anxiety, and autonomic arousal are interrelated with the severity of lower GI symptoms in ASD (17, 18). One miRNA identified in this study, miR-142-5p, has been previously implicated in anxious behavior following prolonged stress (31). Whole blood serotonin levels have also been associated with lower GI symptoms in ASD (29). Here, we identify one miRNA (miR-23a-3p) implicated in ASD-related GI pathology, which has previously been shown to change in depressed patients treated with selective serotonin reuptake inhibitors (32). Specific genes, in particular polymorphisms of the Mesenchymal Epithelial Transition (*MET*) receptor kinase gene, are also associated with GI symptoms in ASD (30). The MET receptor has been shown to modulate miRNA expression (33), and the MET transcript is a putative target of two miRNAs in the present study (miR-23a-3p, miR-23b-3p).

Immunological factors have also been found to be associated with GI symptoms as well as behavior in ASD (34, 35). The unique immunologic patterns associated with ASD may contribute to specific alterations in the microbiome profile that have been reported in children with ASD and GI symptoms (36). Evidence of this nature has even led to efforts at interventions based on the microbiome in ASD (37, 38). In the present study, we identify several miRNAs that are implicated in immune development. For example, miR-28 has been shown to modulate T-cell differentiation and cytokine expression (39), while miR-200a-3p and miR-141-3p have been found to work together to modulate differentiation of interleukin-producing T-helper cells (40). We found minimal overlap between the specific microbes identified in this study, and those of previous GI microbiome studies (36). This may be because the current investigation examines microbial RNA levels in saliva, as opposed to the more traditional 16S approach, using stool samples.

TABLE 3 | Transcripts significantly different between GI phenotypes.

Transcript	*P*-value	Critical value
hsa-miR-28-3p	4.80E-06	0.001056
hsa-miR-1307-5p	8.54E-06	0.002113
hsa-miR-200a-3p	3.33E-05	0.003169
hsa-miR-141-3p	3.77E-05	0.004225
hsa-miR-23a-3p	4.43E-05	0.005282
hsa-miR-23b-3p	5.02E-05	0.006338
hsa-miR-142-5p	1.31E-04	0.007394
hsa-miR-224-5p	1.70E-04	0.008451
hsa-miR-769-5p	3.36E-04	0.009507
hsa-miR-148a-5p	3.74E-04	0.010563
hsa-let-7b-5p	7.27E-04	0.01162
hsa-miR-27a-3p	7.42E-04	0.012676
hsa-let-7a-5p	8.19E-04	0.013732
hsa-let-7c-5p	0.001301	0.014789
hsa-miR-532-5p	0.001603	0.015845
hsa-miR-192-5p	0.002351	0.016901
hsa-miR-186-5p	0.002528	0.017958
hsa-miR-106b-3p	0.003164	0.019014
hsa-miR-200a-5p	0.003643	0.02007
hsa-miR-151a-3p	0.003758	0.021127
hsa-let-7e-5p	0.004643	0.022183
hsa-miR-181a-5p	0.005052	0.023239
hsa-miR-25-3p	0.006292	0.024296
hsa-miR-29c-3p	0.006425	0.025352
hsa-miR-10b-5p	0.00701	0.026408
hsa-miR-22-3p	0.007061	0.027465
hsa-miR-501-3p	0.008192	0.028521
hsa-miR-24-3p	0.009503	0.029577
hsa-miR-27b-3p	0.011937	0.030634
hsa-miR-182-5p	0.0138	0.03169
hsa-miR-3074-5p	0.016482	0.032746
hsa-miR-26b-5p	0.021226	0.033803
hsa-let-7f-5p	0.023545	0.034859
hsa-miR-125b-5p	0.02362	0.035915
hsa-miR-375-3p	0.02457	0.036972
hsa-miR-374a-5p	0.024781	0.038028
hsa-miR-92a-3p	0.029143	0.039085
hsa-miR-148a-3p	0.029199	0.040141
hsa-miR-425-5p	0.029741	0.041197
hsa-miR-222-3p	0.030985	0.042254
hsa-miR-30e-5p	0.035843	0.04331
hsa-miR-30b-5p	0.038612	0.044366
piR-hsa-15023	9.83E-05	8.67E-04
piR-hsa-28405	5.27E-04	0.001734
piR-hsa-17560	0.002069	0.002601
Mycobacterium kansasii	2.13E-06	6.61E-04
Streptomyces albulus	3.08E-04	0.001322
Staphylococcus simulans	8.04E-04	0.001982
Actinomyces radicidentis	0.001163	0.002643
Sneathia amnii	0.001933	0.003304
Lysinibacillus sphaericus	0.002427	0.003965
Candidatus Azobacteroides pseudotrichonymphae	0.002545	0.004626
Cellulomonas gilvus	0.00321	0.005286
Actinobacillus succinogenes	0.003283	0.005947
Capnocytophaga haemolytica	0.00522	0.006608
Corynebacterium singulare	0.006309	0.007269
Streptococcus dysgalactiae	0.007484	0.00793

As further research begins to reveal a clearer understanding of the pathways implicated in ASD patients with GI symptoms, including those specific to the ASD/GI population, we can begin to understand why some patients with ASD appear to respond less reliably to treatment than others with similar GI symptoms (7). Examining the downstream targets of miRNA differentially expressed in those with ASD and GI symptoms would likely contribute substantially to this understanding. Fortunately, with the ability to rapidly, and non-invasively measure miRNA in saliva (21), such information can readily be obtained from large populations. Development of such targeted approaches may provide opportunities for personalized treatment of gastrointestinal symptomatology, and lead to down-stream improvement in related behaviors (7, 10, 11), by impacting anxiety, mood, sleep, and attention (12–16).

This study harnesses, to our knowledge, the largest sample of the salivary micro-transcriptome in ASD. Its inclusion of children with non-ASD developmental delay as part of the control group is a relative strength. Inclusion of participants from multiple geographic sites also promotes generalizability of the findings. However, there are several limitations which should be noted. First, GI disturbances were identified through parent report and review of medical records but were not specifically assessed by physicians as part of the study. Second, we acknowledge that "GI disturbance" is a somewhat artificial distinction that groups together loosely related pathology that occurs in the GI tract. Important physiologic differences exist between conditions such as constipation and reflux, and these underlying biologic differences may have served to enhance false negative findings in the initial analyses. For this reason, we performed secondary analyses of the GI sub-phenotypes. However, this approach also has a trade-off of reducing the study's substantial sample size. Third, there are several pre-analytic factors which may potentially confound this study's findings, including batch effects and sample collection factors. We note that all samples were run on the same sequencing machine, using the same library preparation procedure, performed by the same laboratory technician. Although this analysis did not control for sample collection parameters, such as collection time, prandial status, or prior tooth-brushing, we have previously assessed the impact of many of these factors on the saliva microtranscriptome (21). We note that none of

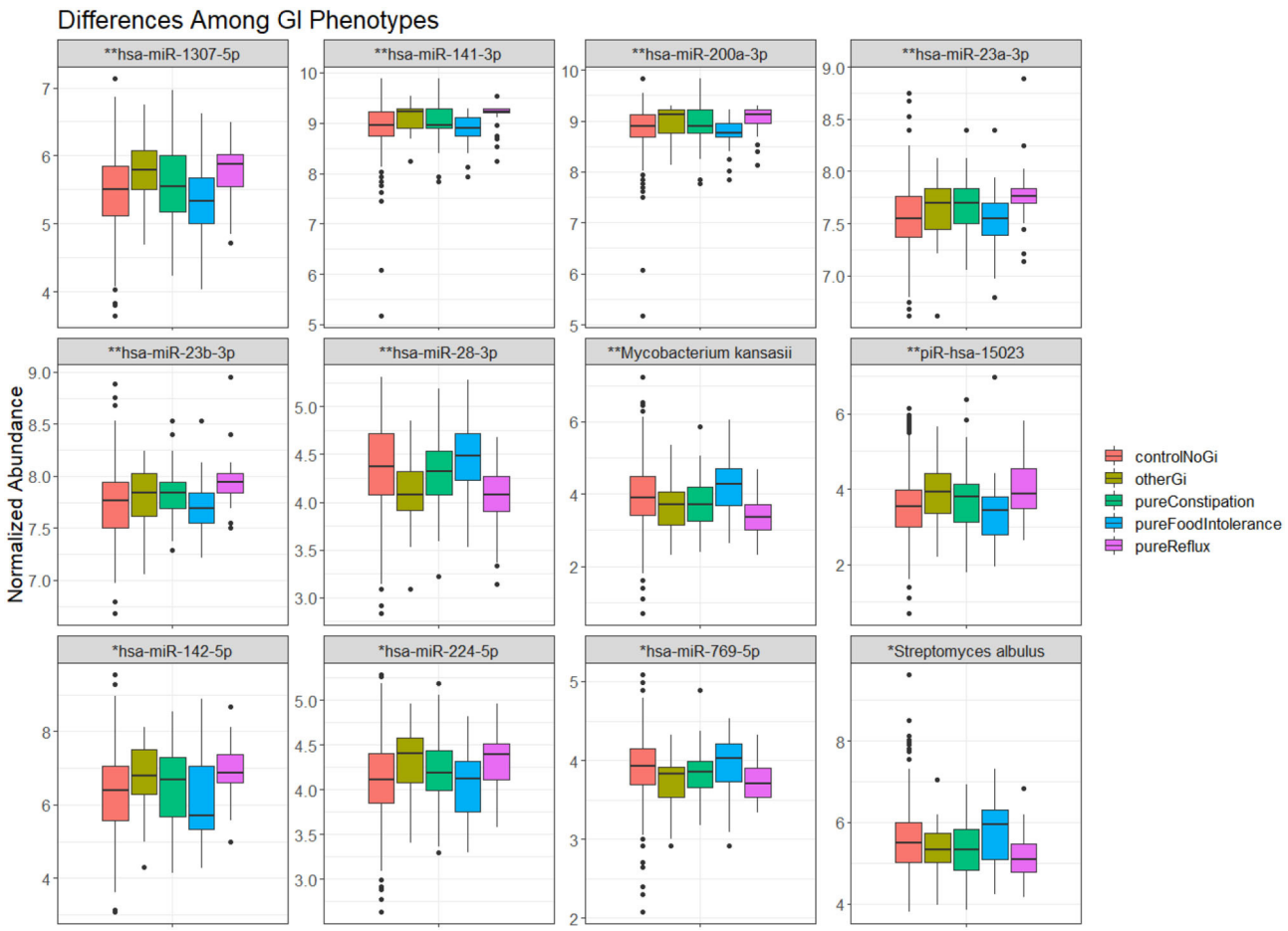

FIGURE 2 | The most significant (12) transcripts differing between GI phenotypes. The whisker box plots represent normalized abundance of miRNAs and microbial RNAs that displayed a significant difference between children without a GI condition (orange), children with constipation (green), food intolerance (blue), reflux (pink), or another GI condition (brown). Significance levels–*p*-val <0.001 (*), *p*-val <0.0001 (**).

TABLE 4 | KEGG pathway enrichment.

KEGG pathway	*p*-value	#Genes	#miRNAs
Steroid hormone biosynthesis	3.04E-12	7	2
Hippo signaling pathway	5.03E-07	15	9
Gap junction	0.001139	10	7
Porphyrin and chlorophyll metabolism	0.005067	9	3
Endocrine and other factor-regulated calcium reabsorption	0.005067	6	7
Drug metabolism–cytochrome P450	0.007162	8	4
Glycosphingolipid biosynthesis–lacto and neolacto series	0.009386	4	5
Ascorbate and aldarate metabolism	0.016999	7	2
Lysine degradation	0.025194	5	7
Proteoglycans in cancer	0.026807	22	9
Thyroid hormone signaling pathway	0.027249	10	7
Long-term depression	0.030698	6	4
Morphine addiction	0.034341	9	7

the microbial features and very few of the miRNA features identified in this study have demonstrated relationships with pre-analytic factors.

We recognize that the *salivary* transcriptome serves as a proxy for the primary pathologic site of most GI disturbances, the lower GI tract. However, several studies have reported

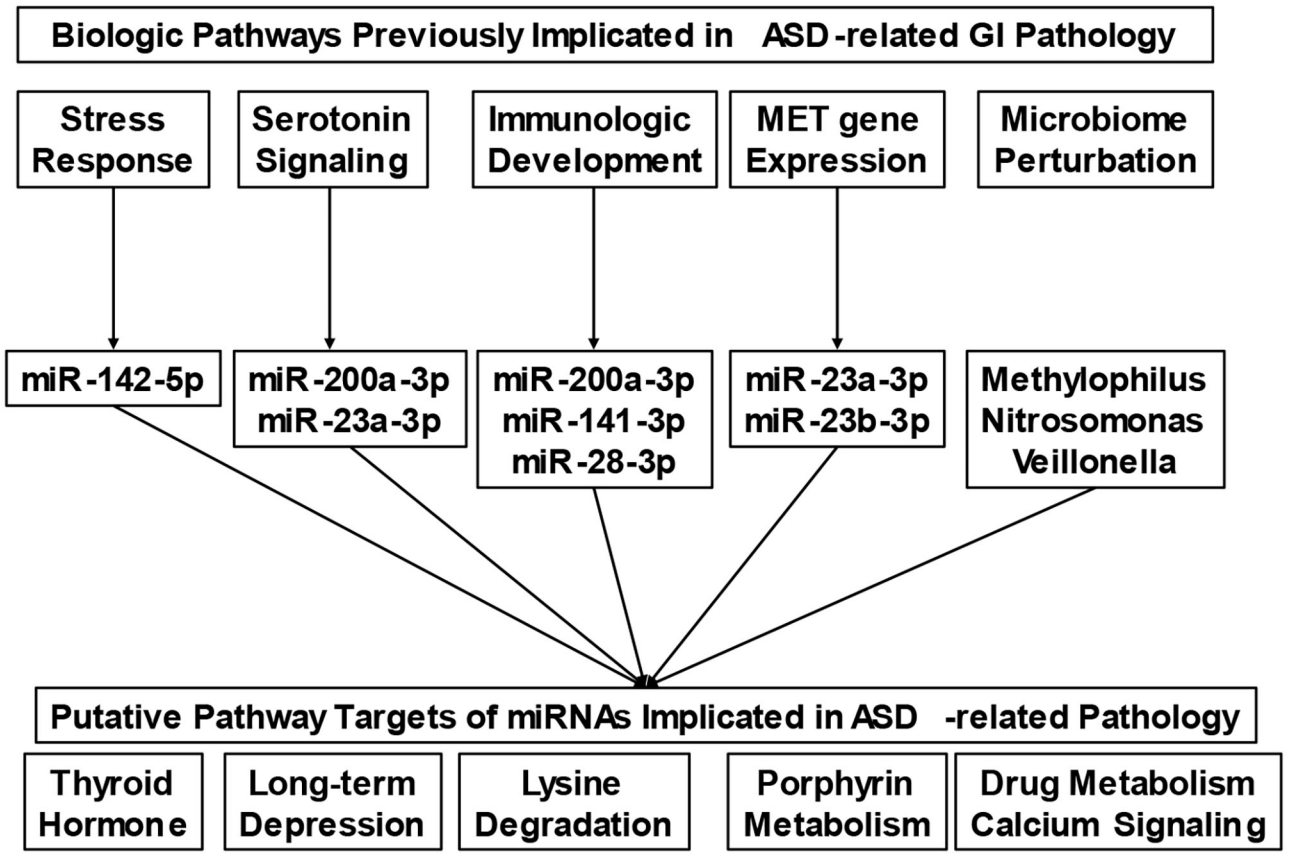

FIGURE 3 | Putative micro-transcriptome links between biologic mechanisms and physiologic pathways involved in gastrointestinal pathophysiology. The conceptual diagram connects biologic pathways that have been previously implicated in Autism Spectrum Disorder (ASD)-related gastrointestinal (GI) pathology with putative micro-transcriptome features (i.e., microRNAs and microbes). Micro-transcriptome features were included based on their associations with the biologic pathways of interest in the existing literature. Collectively, the microRNAs display target enrichment for several clinical findings (i.e., thyroid hormone signaling, long-term depression, etc.), that are known contributors to GI disturbance.

significant overlap between saliva and stool micro-transcriptome features (41–43). Unlike the stool microbiome, we note that the saliva microbiome can be repeatedly sampled on demand and has shown resilience to antibiotic treatments (44). These characteristics make saliva an attractive source for sampling GI-related biology (particularly in patients with conditions such as reflux, eosinophilic esophagitis, or cyclic vomiting). Our previous work with parents of children with ASD has shown that they overwhelmingly prefer saliva as a clinical biofluid (45). Additionally, we note that association analyses between salivary transcriptome elements and ADOS scores rely solely on ASD and DD participants for whom these assessments were available. The lack of TD participants in this analysis could have impacted the findings. Finally, we must also consider the possibility that some of these markers may be caused by the downstream effects of the gastrointestinal symptoms or treatments, rather than serving a mechanistic role, but this would not diminish their potential use as biomarkers.

This is, to our knowledge, the first effort to examine the salivary RNA profile associated with GI symptoms in ASD, in a large population study. With the increased understanding of the critical importance of subtyping for meaningful precision medicine approaches in ASD (20), as well as the importance of GI symptomatology in behavioral issues in ASD (7, 10, 11), and the potential for mechanistic understanding through examination of the downstream targets of differentially regulated miRNAs, this is an important future direction of investigation. A subset of the micro-transcriptome features identified in this study displays relationships with treatment modality and are associated with autistic behaviors. The pathobiologic targets of these micro-transcriptome markers may serve as novel targets for individualized therapeutic interventions aimed at easing pain and behavioral difficulties seen in ASD-related GI disturbance.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available due to the proprietary nature of the research. Requests to access the datasets should be directed to David Levitskiy, david.levitskiy@motionintel.com.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Western Institutional Review Board (IRB #20180172). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

FM, RG-K, RS, DB, KS, and SH contributed to conception and design of the study. DL performed the statistical analysis. AC and PT contributed to data collection and formatting. DB, KS, and SH wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpsyt.2021.824933/full#supplementary-material

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The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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SYSTEMATIC REVIEW
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Examining Effectiveness and Predictors of Treatment Response of Pivotal Response Treatment in Autism: An Umbrella Review and a Meta-Analysis

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The current study aimed to provide a comprehensive appraisal of the current evidence on the effectiveness of Pivotal Response Training (PRT) for individuals with autism spectrum disorder (ASD) and to explore predictors of treatment response. We conducted a systematic review of the following electronic databases and registers: PsycINFO, Medline, Embase, Cochrane Central Register of Controlled Trials, ERIC, Linguistics and Language Behavior Abstracts. Six systematic reviews were identified, two with meta-analytic component. Identified reviews varied widely in terms of their aims, outcomes, and designs which precluded a unified and consistent set of conclusions and recommendations. Ten RCTs were identified. Eight of identified RCTs reported at least one language and communication-related outcome. Statistically significant effects of PRT were identified across a majority of identified RCTs for a range of language and communication skills. However, evidence for positive treatment effects of PRT on outcome measures assessing other domains was less robust and/or specific. Overall, both previous systematic reviews and new meta-analysis of the RCTs suggest that PRT shows promise for improving language and communication. Only four RCTs examined the association between baseline child characteristics and treatment outcomes, however, no consistent pattern emerged. This review has identified several key methodological and design improvements that are needed to enable our field to fully capitalize on the potential of RCT designs and characterize detailed profiles of treatment responders. These findings are essential for informing the development of evidence-based guidelines for clinicians on what works for whom and why.

Keywords: social deficits, language, children, umbrella review, randomized controlled trial, pivotal response treatment, meta-analysis, predictors of outcomes

INTRODUCTION

Autism spectrum disorder (ASD) is a cluster of neurodevelopmental disorders characterized by social and communication impairments and the presence of restricted and repetitive patterns of behavior and interests (1). In addition to core symptomatology, a significant portion of individuals with ASD experience a range of additional neuropsychiatric and neurodevelopmental symptoms, cognitive deficits and medical comorbidities (2–6). Although some individuals with ASD have good long-term outcomes (7), a majority continue to experience poor mental health and quality of life with unsatisfactory social, educational and vocational outcomes (8–10). Given the high prevalence, life-long nature and significant public and health costs (11, 12), the development of effective and empirically supported treatment approaches is a crucial priority. Furthermore, where potentially effective interventions are available, a state of the art summaries and critical appraisals of existing evidence is critical for informing and guiding clinical and policy-related decision-making. In addition to establishing an evidence base for the effectiveness of specific treatments, as a necessary step on the path to precision medicine, it is also crucial to understand and characterize profiles of children who stand to benefit the most from a particular treatment, and of children who are unlikely to show significant gains.

Early and intensive interventions based on applied behavior analysis (ABA) and delivered in structured settings have been shown as effective for teaching specific functional skills, reducing problem behaviors, and improving language and intellectual functioning (13–18). However, highly structured ABA approaches may be limited by a lack of generalization of acquired skills (17), high financial cost and time-consuming nature. These concerns have led to the emergence of a group of interventions commonly referred to as the Naturalistic Developmental Behavior Interventions (NDBIs) (19) that combine key ABA principles and techniques with the child-led developmental approach incorporating motivational variables delivered in naturalistic, everyday settings.

Pivotal Response Treatment (PRT) (20, 21) is an NDBI developed to target pivotal areas of motivation, self-initiations, self-management, and responding to multiple cues through the combination of operant learning contingencies, motivational teaching strategies, and child-driven approaches. The rationale behind focusing on noted core developmental areas is that if successfully targeted, they can have a positive effect on a range of other, more specific skills and behaviors (22, 23). Similar to other NDBIs such as the Early Start Denver Model (ESDM) (24), PRT teaching strategies are rooted in the ABA approach and embedded within naturalistic child-adult interactions and designed to enable children to benefit from typical pathways that would not be otherwise available due to the core ASD impairments such as lack of social motivation and attention. One of the key components of the PRT is active parental participation (20) which has been suggested as crucial not only for increasing the number of learning opportunities and overall treatment intensity (25) but also for promoting generalization (26) and beneficial effects on parental well-being (27).

A number of single-subject, small *N* and non-randomized group-based studies have suggested the effectiveness of PRT in ASD (28). For instance, PRT has been shown as effective for improving specific commutation skills such as question-asking, number and length of utterances, speech intelligibility, and spontaneous language, conversation, play, and social initiations (29–32). Furthermore, several studies indicated that PRT led to a reduction in disruptive behaviors (33), anxiety (34) and repetitive behaviors (35).

The lack of randomized controlled trials (RCTs) has been identified as one of the key barriers for progressing the science of ASD behavioral intervention in general (36), and for PRT in particular (37). Therefore, the emergence of PRT RCTs in the last 5 years has been a positive development. It is particularly encouraging that recent RCTs have suggested that PRT outcomes are quite favorable in certain symptom and functional domains, in particular with regards to increase in expressive and receptive language (38, 39) and adaptive communication skills (38). Potentially promising findings for improvements in cognitive functioning (40) and reduction in overall ASD severity (39, 40) also emerged.

Given the increase in the adoption of PRT in clinical practice (21), it is important to systematically appraise existing evidence and achieve a current consensus on the effectiveness of PRT for specific outcomes. It is also crucial to go beyond appraising evidence for group-level effectiveness and provide an in-depth characterization of the baseline characteristics that are associated with positive treatment outcomes. Further, it is important to identify the limitations of the current PRT treatment literature and highlight crucial areas for future improvements. Therefore, we aimed to provide an accessible, state-of-the-art synthesis and integration of current findings on PRT in ASD. The first aim was to conduct an umbrella review of previously published systematic examinations of the literature on the effects of PRT. Although all research designs provide important evidence for the effectiveness of particular treatment practices, RCTs are best equipped for estimating the potential benefits of specific interventions. Crucially, in addition to estimating average treatment effects, if well-powered, RCTs can identify predictors of treatment response and why particular individuals benefit from specific interventions (41, 42). Therefore, the current study aimed to conduct a meta-analysis of PRT RCTs published to date. More specifically, we aimed to (i) investigate the effectiveness of PRT in the domains of core ASD (overall ASD severity, restricted and repetitive behaviors, social and communication abilities) and related (language, cognitive functioning, adaptive behavior, and co-occurring symptoms and behavioral problems) outcomes, and (ii) if enough data were available, to examine predictors of treatment outcomes.

METHODS

Review methodology adhered to the steps described in the Preferred Reporting Items for Systematic reviews and Meta-Analyses (PRISMA) statement (43).

TABLE 1 | Search terms by domain.

Category	Search terms
Population	autism, autistic, Asperger*, asd, pervasive development*, pdd, pddnos
PRT related	Pivotal, prt, naturalistic, communication*, development, language*, self, self directed, initiat*, manag*, responsiv*, social, behavior*, behavior*, skill*, parent, parents, parental
Treatment	teach*, paradigm*, intervention*, treatment*, approach, therap*, training, learning
RCT	random*, rct, clinical trial*, controlled trial*, placebo, blind*, doubleblind, quasirandom*, control group*

Abbreviated search term.

Information Sources and Search Strategy

Searches were performed in PsycINFO (Ovid) (to May Week 3 2020), Medline (Ovid) (to May 20th, 2020), Embase (Ovid) (to May 20th, 2020), Cochrane Central Register of Controlled Trials (Ovid) (to April 2020), ERIC (Ebsco) and Linguistics and Language Behavior Abstracts (Proquest) by a librarian (PC). All Ovid database searches were conducted on 22nd May 2020 with the ERIC and LLBA searches subsequently run on 25th May 2020. Search terms were developed based on (i) a literature search on ASD and pivotal response treatment and (ii) consultations with the experts in the field and included terms around the broader category of language and behavioral skills training. The broader literature on parental interventions was also examined. No specific subject heading for pivotal response treatment was identified in the included databases, however, the search was made broad by including any mention of the term pivotal. A broad limit was applied to select randomized controlled trials only. The PsycINFO search (**Supplementary Table 1**) was adapted to the other databases with specific limits and replacement of proximity search operators with Ebsco and Proquest systems. **Table 1** shows the key search terms that were used.

Eligibility Criteria

Articles published in English were included if they were (a) empirical studies evaluating PRT (manuals and commentaries were excluded but their reference sections were reviewed for relevant empirical papers), (b) published in peer-review journals (conference abstracts and theses were excluded), (c) Randomized controlled trials (RCTs) (other designs including non-randomized studies, controlled before-and-after studies, quasi-experimental and case studies were excluded), and (d) included individuals with ASD (including autism, Asperger's disorder or pervasive developmental disorder not otherwise specified [PDD-NOS] with and without an intellectual disability). No age nor setting (e.g., home, school/kindergarten/other education setting, clinic) limits were imposed. Systematic reviews were excluded from the meta-analysis component but identified for inclusion in the umbrella review component of the study. Reviews without the systematic component were excluded.

Study Selection and Data Extraction

Following the initial database search, duplicates were removed and study titles were reviewed to remove obviously irrelevant articles. Abstracts of candidate articles were then reviewed for potential inclusion for a full review. Inclusion at this stage only required that the article described a study or review of PRT and ASD. Identified abstracts were removed if they clearly did not meet the inclusion criteria or met one of the exclusion criteria (e.g., single case study, non-randomized trial, etc.). The remaining articles were reviewed in full and evaluated for inclusion/exclusion. The reference sections of the articles were also screened to identify additional articles that might have been missed. Articles that did not meet the inclusion criteria were flagged for removal. Retained PRT RCTs and systematic reviews were coded for the following information:

Umbrella Review: (a) type (meta-analysis and qualitative), (b) inclusion/exclusion criteria, (c) period captured, (d) study aims, (e) whether reviews appraised the quality of the included empirical studies, (f) characteristics of included studies (e.g., total *N*; *N* of participants; age), (g) findings regarding outcomes and whether reviews appraised moderators and mediators of treatment outcomes, and (h) specification of limitations of the current research and future directions.

Empirical Studies: (a) participant characteristics for both PRT and control groups (e.g., *N*; age; sex, ethnicity, (b) inclusion/exclusion criteria, (c) completion rates, (d) intervention characteristics for both PRT and control group (where applicable) including setting, duration and intensity, (e) dependent variables (primary and secondary), (f) fidelity, (g) outcomes, (h) predictors of treatment outcomes, and (i) country where the study was conducted. Outcomes were narratively summarized and relevant information for the meta-analysis was extracted (for further detail see Analytic Strategy subsection below). Quality indicators for studies and outcomes included in the meta-analysis were assessed based on the assessment protocol utilized by Sandbank et al. (44). Study level indicators included assessment for selection bias (random assignment), blinding and attrition. Measurement level indicators included (i) proximity—whether outcomes were directly taught by the intervention (proximal outcomes) or were developmentally downstream from what was taught by the intervention (distal outcomes), (ii) context—whether assessments were conducted in the same context where the interventions are delivered, for instance, the use of similar materials for both the intervention and the assessment (context-bound) or in the context different from the intervention in terms of setting, assessor or material (generalized), and (iii) the presence of correlated measurement error (CME) which occurs in situations when parents or teachers who deliver interventions also participate in outcome assessments.

Analytic Strategy

A meta-analysis was performed to consolidate studies examining the effect of PRT on a variety of dependent variables. Where these measures were comparable but not identical, the reported statistics were standardized to facilitate the combination and comparison of the effect estimates. Given that all studies used a

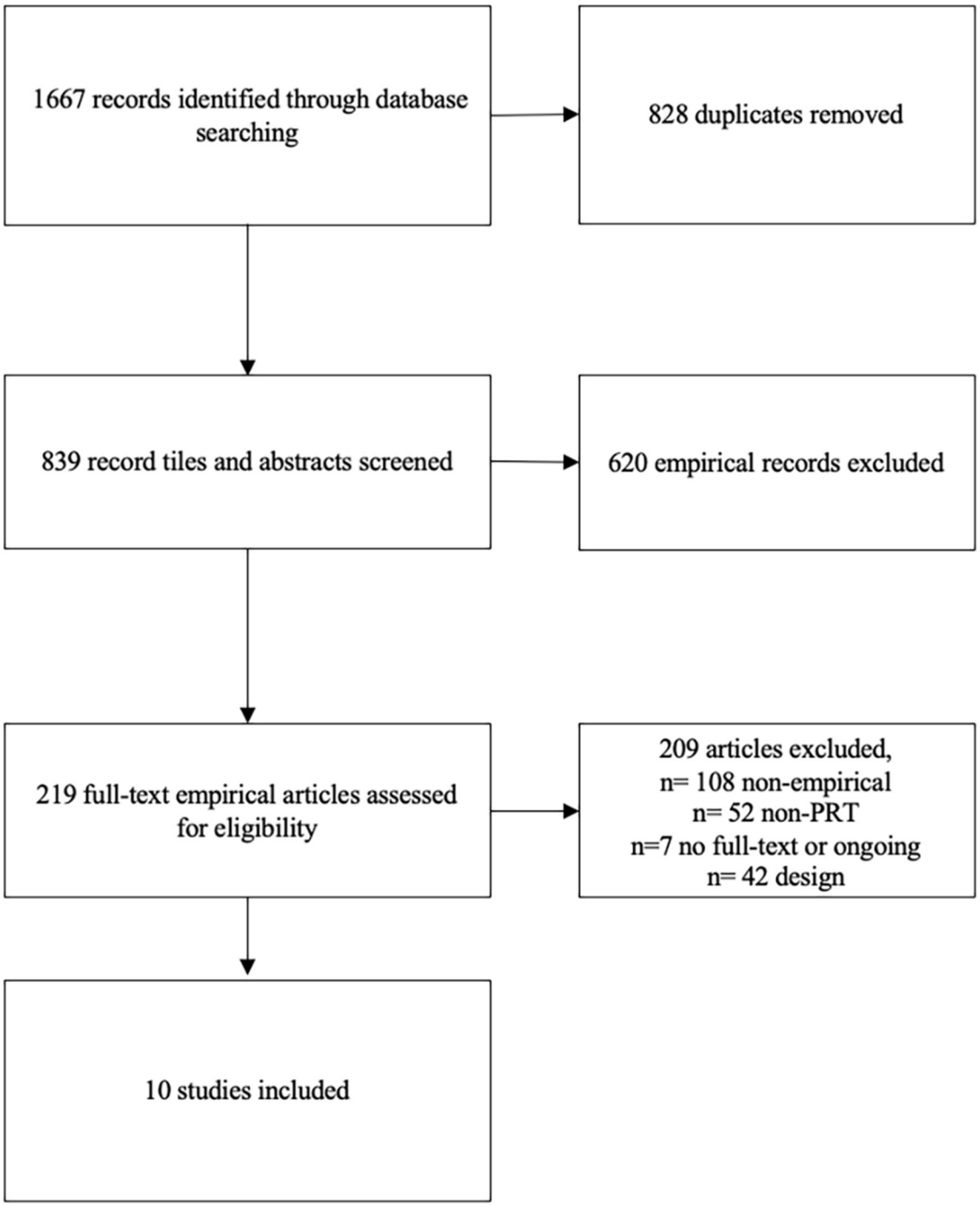

FIGURE 1 | PRISMA flow diagram.

baseline/follow-up design and only the mean, standard deviation and sample size were available, a conservative correlation coefficient of 0 (between time points, within pairs) was used—although it is acknowledged that, in practice, this figure is likely to be higher, which would result in increased power and narrower confidence intervals. After a consolidated mean and standard deviation were produced for the treatment and control groups, we used a Student's *T*-Test to determine if there was a significant difference between the PRT treatment group and the control treatment group. A funnel plot and Egger's

test of a small study bias were used to examine the evidence of publication bias. Meta-regression was used to investigate the contribution of study-level and quality indications on treatment outcomes.

RESULTS

Study Selection

Figure 1 provides an overview of the search results at each stage of the process. Six systematic reviews specifically focusing on the PRT were identified. A Cochrane Systematic Review was also identified (45), however, it was not included as it was still at the protocol stage. Ten RCTs met all the inclusion criteria.

Umbrella Review

Two reviews included a meta-analytic component (28, 46) and four provided a narrative summary of the studies (37, 47–49). **Table 2** provides an overview of the characteristics and findings of the identified reviews.

Bozkus-Genc and Yucesoy-Ozkan (28) focused on meta-analytically appraising findings from 34 single case studies to evaluate the effectiveness of PRT across a range of outcomes and identify potential moderators of treatment outcome. Authors found that at least 70% of studies were labeled as highly or fairly effective across dependent variables, irrespective of the method for estimating effect size (e.g., percentage of non-overlapping data [PND], percentage of non-overlapping corrected data [PNCD], and percentage of data points exceeding median [PEM]). Despite positive findings, Bozkus-Genc and Yucesoy-Ozkan (28) also identified a number of methodological limitations. More specifically, treatment integrity, maintenance/generalization, and social validity data were included in only 44, 50, and 25% of studies, respectively.

Ona et al. (46) utilized a meta-analytical approach to evaluate social interaction, communication and repetitive behaviors (RRB) outcomes of 7 studies published before August 2017. The authors were able to synthesize findings for only expressive language and communication outcomes. This analysis supported statistically significant benefits of PRT over control condition for expressive language (2 studies, direct observation; standardized mean difference [SMD]: −0.57, 95% CI 0.04, 0.93, $p = 0.03$), but not for adaptive communication (2 studies, parent and clinician report; SMD: 1.12, 95% CI −0.49, 2.73, $p = 0.17$). At the individual study level, there was evidence for statistically significant benefits of PRT over control condition for RRB (direct assessment) (33) and social interaction clinical global impression-improvement [CGI-I] (38) but not for receptive language (38), communication (subjective report) (53) nor several parental or clinician report measures of expressive language including Vineland Adaptive Behavior Scales (VABS) and MacArthur-Bates Communicative Development Inventories (CDI) (38, 53). The quality of evidence for outcomes was rated as very low for communication and low across other outcomes, based on the Grading of Recommendation, Assessment, Development and Evaluation (GRADE) (54) approach. Several areas for improvement were noted, including the need for a more detailed assessment of implementation fidelity, increased use of validated and objective outcome assessment methods and inclusion of broader outcomes, in particular quality of life and parental stress.

Examining a range of different treatment designs published up to June 2014; Verschuur et al. (37) explored PRT effectiveness for improving children's outcomes, parental and staff outcomes and skills, and evaluated quality and certainty of evidence. Out of 37 identified studies, 35 targeted child behaviors and skills (17 self-initiation, 1 motivation, 31 communication and language skills, 6 play skills, 5 adaptive functioning, 5 maladaptive behaviors, 4 ASD symptom severity, 3 affect, 2 cognitive functioning, 2 academic functioning, 1 face processing, 1 attendance and compliance), 13 targeted parental behaviors (9 implementation fidelity, 2 stress, 2 affect, 2 self-efficacy, 1 empowerment) and 7 staff skills (6 implementation fidelity, 1 effectiveness of training on the ability of staff to conduct assessments). Verschuur et al. found that (i) 43.6% of studies showed conclusive or preponderant evidence that PRT increases self-initiations and results in collateral improvements in communication and language, play skills, affect and reductions in maladaptive behavior, (ii) majority of caregivers and staff members were able to implement PRT techniques, and (iii) collateral improvements in caregivers' and staff members' behaviors were appraised by only a few studies and evidence was qualified as sparse. A number of important areas for improvement was indicated including: (i) need for more experimental and RCT designs, (ii) need for more stringent operationalization and measurement of pivotal skills and collateral outcomes, (iii) characterizing predictors of treatment outcomes and understanding active ingredients of PRT, (iv) understanding parental and staff predictors of effective treatment implementation, and (v) identification of the most effective formats of parental and staff training.

Two systematic reviews appraised evidence of PRT effectiveness for improving communication and/or social skills. Forbes et al. (49) focused on experimental designs by evaluating primary linguistic and verbal behavior outcomes. Boudreau et al. (47) examined peer-mediated PRT for facilitating social-communication behaviors. Interestingly, Forbes et al. (49) noted that the majority of 50 identified studies did not report sufficient detail to enable evaluation of the linguistic forms or verbal behavior functions. Across identified studies, there was evidence for the generalization of communication skills to untargeted people, settings, materials, and/or activities, however, none of the studies described results that indicated improved generalized and collateral verbal behavior function. Using a modified framework for appraising the quality of evidence by Reichow et al. (50), Boudreau et al. (47) concluded that none of the 5 identified studies (10 participants in total across studies) met the criteria for classification as promising or established evidence-based practice for improving social-communication impairments.

Finally, a review by Cardogan and McCrimmon (48) evaluated adherence of 17 identified PRT studies to specific research quality standards selected by authors based on a range of existing quality frameworks. They found that studies showed good quality

TABLE 2 | Overview of the characteristics and findings from the identified systematic reviews.

Review	Type	Inclusion criteria	Period	Outcomes	Aims	Study quality	Study characteristics	Findings summary
Boudreau et al. (47)	NQ	Peer-Mediated; Age range 4–18 years; Design: no design restrictions	NR	SC	Evaluate peer-mediated PRT for facilitating the SC of school-aged children with ASD	Modified/expanded (by authors) framework for appraising the quality of evidence Reichow et al. (50)	*N* = 5 studies; *N* = 10 participants (8 after removing overlap); Age: 7–10 years; IQ: 55–85	None of the studies met the criteria for classification as promising or established EBP for improving SC impairments
Bozkus-Genc and Yucesoy-Ozkan (28)	M	Design: Single-case; Age range: 1–13 years	1979–2012	No restrictions	Evaluate participant and intervention characteristics, effectiveness and moderators	NA	*N* = 34 studies; Age: 2 years, 5 months-12 years, 8 months; Settings: multiple (44.1%), clinic (26.4%), school (14.7%), home (8.8%), community (5.8%)	Mean PND: 76.10% (SD = 33.65, range: 0–100); effect sizes > 90% in 38.2% of studies, 70–89% in 33.4% of studies, and < 70% in 29.4% of studies; PND scores >70% for all of the dependent variables except play and social skills. 14 studies labeled as highly effective, 11 fairly effective, 10 questionable/ineffective. Mean PNCD: 78.03% (SD = 34.38, range: 0-100); effect sizes > 90% in 41.1% of studies, 70–89% in 26.5% of studies, and < 70% in 26.4% of studies; PNCD scores >70% for all of the dependent variables except play skills. 14 studies labeled as highly effective, 9 fairly effective, 10 questionable/ineffective. Mean PEM: 89.34% (SD = 22.18, range: 0-100); effect sizes > 90% in 79.4% of studies, 70–89% in 11.7% of studies, and <70% in 8.8% of studies; PEM scores >70% for all of the dependent variables. 27 studies labeled as highly effective, 4 fairly effective, 3 questionable/ineffective.
Cardogan and McCrimmon (48)	NQ	<18 years of age	NR	Study quality	Evaluate adherence of PRT studies to specific research quality standards	Seven specific standards chosen by authors	*N* = 17 studies	Systematic application of an intervention procedure: five studies utilized a pre–post evaluation, 11 multiple baseline procedure, one did not collect any baseline data; Comparison of intervention approaches: two studies compared intervention approaches; Use of standard intervention protocols: 9 studies explicitly aligned with the PRT manuals;

(Continued)

TABLE 2 | Continued

Review	Type	Inclusion criteria	Period	Outcomes	Aims	Study quality	Study characteristics	Findings summary
								Treatment fidelity: two studies adhered to the recommended fidelity standard prior to the study start but there were variations during the intervention, five studies no reference to the fidelity measures; Use of objective evaluators: 11 studies used objective evaluators, remaining studies did not reference the use of objective evaluators (two did not require it given the design); Inter-rater reliability: 12 studies reported some form of inter-rater reliability; Longitudinal studies: 8 studies collected follow-up data after the post-treatment stage and 9 did not.
Forbes et al. (49)	NQ	Design: Experimental; Other: at least one communication skill as a dependent variable	1987–2018	Communication	Evaluate primary linguistic and verbal behavior outcomes following PRT and how generalized and collateral outcomes were reported	NA	$N = 50$ studies	The majority of studies aggregated results and/or did not report sufficient detail to determine linguistic forms and/or verbal behavior functions; There was evidence for the generalization of communication skills to untargeted people, settings, materials, and/or activities; Only one study indicated untargeted linguistic forms emerged following PRT and none of the studies described results that indicated improved generalized and collateral verbal behavior functions.
Ona et al. (46)	M	Design: RCT; Age range: ≤ 18 years of age	up to August 2017	SC, SI, RRB	Evaluate social communication, social interaction, and repetitive behavior outcomes in PRT RCTs	GRADE	$N = 7$ studies; $N =$ 181 participants; Age: 2.4–9.2 years	Communication (subjective report): two studies, SMD 1.12 (95% CI −0.49; 2.73), $p = 0.17$, GRADE: very low; Expressive language (subjective report): one study, SMD 0.45 (95% CI −0.13; 1.03), $p = 0.13$, GRADE: low; Expressive language (direct measurement): two studies, SMD 0.48 (95% CI.04; 0.93), $p = 0.03$, GRADE: low;

(Continued)

TABLE 2 | Continued

Review	Type	Inclusion criteria	Period	Outcomes	Aims	Study quality	Study characteristics	Findings summary
								Receptive language (subjective report): one study, SMD 0.22 (−0.35; 0.79), $p = 0.45$, GRADE: low; Social Interaction: one study (subjective report): SMD 0.48 (−1.10; 1.06), $p = 0.10$, SMD 0.46 (−0.12; 1.04), $p = 0.12$ for CGI-S and SMD 1.12 (0.50; 1.74), $p = 0.0004$ for CGI-I Repetitive Behaviors (direct assessment): one study, SMD 15.97 (95% CI 11.57 to 20.36) p <0.0001, GRADE: low.
Verschuur et al. (37)	NQ	Age: no constraints; Design: no constraints	Up to June 2014	No restrictions	Evaluate: the range of targeted skills; PRT effectiveness for improving children's outcomes; PRT effectiveness for improving parental and staff outcomes and skills; the certainty of evidence; identify limitations and future directions	Quality of evidence (51); Certainty of evidence following classification by Ramdoss et al. (52) into suggestive, preponderant and conclusive	$N = 37$ studies $N =$ 420 participants; Age: 1–12.7 years	56.4% of studies had serious methodological limitations; 43.6% of studies showed conclusive or preponderant evidence that PRT increases self-initiations and results in collateral improvements in communication and language, play skills, affect and reductions in maladaptive behavior; The majority of caregivers and staff members were able to implement PRT techniques; Few studies reported on collateral improvements in caregivers' and staff members' behaviors and evidence was qualified as sparse.

GRADE, Grading of Recommendation, Assessment, Development and Evaluation; M, meta-analysis; NQ, non-quantitative; NR, not reported; PEM, percentage of data points exceeding median; PNCD, percentage of nonoverlapping corrected data; PND, percentage of nonoverlapping data; PRT, Pivotal Response Training; SC, social and communication; SI, social interaction; SMD, standardized mean difference.

benchmarks with regards to the use of standardized treatment protocols and application of treatment procedures, inter-rater reliability and objective evaluators. However, variable quality of adherence to treatment fidelity (only 2 studies), comparison of PRT to other approaches (only 2 studies) and collecting follow-up data after the post-treatment stage (8 studies) was observed.

In summary, the reviews undertaken to date covering the period up to 2018 indicate that although PRT can be effective across a range of language and communication outcomes, evidence for other symptom domains and behaviors is limited and that the previous research quality was adversely affected by a range of factors. Importantly, despite the strengths of the previous systematic reviews, they have varied widely in terms of their focus (both with respect to outcome and design) and only two of the reviews included a meta-analytic component and only one focused on RCTs (46). Although the meta-analysis by Nordvik Ona and colleagues was published relatively recently, this review did not capture RCTs published after 2018 and was only able to conduct three meta-analyses, each with only two studies and included one unpublished study (55). Crucially, none of the identified reviews specifically focused on identifying predictors of treatment outcomes. Therefore, it is difficult to form a comprehensive picture of the current state of the literature and the strength of the existing evidence-base for PRT in ASD. Given that four recently published RCTs were not included in any of the summarized systematic reviews, conducting an updated meta-analysis has the potential

TABLE 3 | Overview of the characteristics and findings from the identified randomized controlled trials.

Study	Participants		Intervention		Dependent Variables	Outcomes	Predictors
	PRT	Other	PRT	Other			
Barrett et al. (56)	$N = 12$; $M_{age} =$ 35.75 mths, SD = 9.31; 8.33% Female; Ethnicity: White (75%), Latino (17%), Asian (8%), Multi-racial (0%).	$N = 9$; $M_{age} =$ 38.22mths, $SD =$ 9.78; 11.1% Female; Ethnicity: White (45%), Latino (22%), Asian (11%), Multi-racial (22%).	PRISM Model: Setting: clinician delivered plus parental component; Duration: 6 mths Intensity: up to 10 hrs/w (8 hrs clinician one-on-one; 2 hrs parent education); Mean intensity = 6.81 hrs (25% families met the threshold of 80% completion of all possible treatment hours).	Waitlist	Parent-child play interaction coded for: (i) Parent social bids; (ii) Child social responsiveness; (iii) N total words; (iv) N different words; (v) MLU.	(i) Parent social bids: no significant changes; (ii) Child social responsiveness: significant improvement in PRT (an increase from responsive to 67% of opportunities pre- to 80.9% post-treatment) but not waitlist group; (iii) and (iv) N total and different words: not a significant increase in PRT group and no changes in waitlist; (v) MLU: significant increased in PRT but not waitlist group.	The minimally verbal subgroup ($N = 5$) showed large effect sizes (but not statistically significant) for all pre- to post-treatment comparisons. Although at the level of total PRT group initial child responsiveness with caregivers did not show significant association with any of the subsequent outcomes, it was significantly associated with gains in total words, and although no reaching statistical significance, it was moderately associated with gains in different words and mean length of utterance.
de Korte et al. (57)	$N = 22$; $M_{age} =$ 11.87 yrs, $SD =$ 1.62; 27.3% Female; Ethnicity: not reported.	$N = 22$; $M_{age} = 11.70$ yrs, $SD = 2.11$; 31.8% Female; Ethnicity: not reported.	PRT: Setting: seven parent-child sessions, three parent-only sessions, two sessions with involvement of the teacher; Duration: 12 weeks; Intensity: 45 mins per sessions, 90 min per sessions where teachers were involved.	TAU.	Primary: SRS total score; Secondary: CGI; ADOS-2; VABS ABC and subscale scores; Brief Problem Monitor-Parents; Parenting Stress Questionnaire.	(i) SRS total score: significantly higher reduction in PRT vs. TAU on parent-report but not teacher report; (ii) Proportion of responders on CGI-I higher in PRT vs. TAU, however, NS at 12-week and reaching significance at 20-week follow-up (but NS after correction for multiple comparisons); (iii) ADOS-2: NS between PRT vs. TAU; (iv) VABS: NS for VABS ABC, significant improvement in socialization score in PRT vs. TAU; (v) Brief Problem Monitor-Parents: significantly higher reduction on total score in PRT vs. TAU; (vi) Parenting Stress Questionnaire: NS between PRT vs. TAU.	No significant correlations between age, sex and IQ with SRS outcomes; lower symptom severity on ADOS CSS total score associated with higher improvements in the SRS-2 scores in PRT (but not TAU) group.

(Continued)

TABLE 3 | Continued

Study	Participants		Intervention		Dependent Variables	Outcomes	Predictors
	PRT	Other	PRT	Other			
Gengoux et al. (39)	$N = 23$; M_{age} = 49.5 mths, SD = 11.2; 9.5% Female; Ethnicity: White (26%), Latino (17%), Asian (8.7%), Multi-racial (4%), Other (8%).	$N = 20$; Mage= 47.2 mths, SD = 10; 15% Female; Ethnicity: White (30%), Latino (5%), Asian (60%), Multi-racial (0%), Other (5%).	PRT-P: Setting: clinician in home-delivered plus parental component; Intensive phase: Duration: 12 weeks; Intensity: 10h/pw in home clinician delivered; 1h/pw parent training; Maintenance phase: Duration 12 weeks; Intensity: 5h/pw in home clinician delivered; 1h/pm parent training.	DTG	Primary: N functional utterances during 10-min SLO (baseline, week 12 and 24); Secondary: BOSCC; CDI; VABS; PLS-5; MSEL; SRS-2; CGI-S and CGI-I.	Primary: Significantly higher increase in the number of utterances in PRT vs. DTG at both 12 and 24 weeks (primarily driven by the nonverbally prompted utterances); Secondary: Significant treatment effect for BOSCC total and SC scores, CDI (words produced out of 396 and 680), CGI-S, CGI-I (24 months); No treatment effects for PLS-5, MSEL, SRS-2 and VABS.	SLO: age, sex, and baseline characteristics did not predict treatment response; BOSCC: total score: association with lower MSEL scores (predominantly NVIQ).
Hardan et al. (38)	$N = 25$; M_{age} == 4.1 yrs, $SD = 1.2$; 24% Female; Ethnicity: not reported.	$N = 23$; $M_{age} = 4.1$ yrs, $SD = 1.3$; 6 Female; Ethnicity: not reported.	PRT-G; Setting: parent delivered; Duration: 12 weeks; Intensity: Eight 90 minute visits (4-6 parents, 1–2 clinicians); Four visits-parent-child dyads with a clinician (60 min).	PEG Duration: 12 weeks; Intensity: Ten 90 minute visits (4-6 parents, graduate student); Two visits-parent-child dyads with a psychologist (60 min).	Primary: N of functional utterances during 10-minute SLO (baseline, week 6 and 12) Secondary: CDI; VABS; CGI-S and CGI-I; SRS; PLS-4.	Primary: In both PRT-G and PEG groups significant improvements in the total number of utterances, improvement higher in PRT-G vs. PEG; Treatment effect most pronounced for imitative and non-verbally prompted utterances, NS for unintelligible and verbally prompted utterances; Fidelity modified treatment effects for total and imitative but not verbally, nonverbally prompted and spontaneous utterances. Secondary: Significant treatment effect for VABS Communication (expressive and receptive) scores, CGI-S and CGI-I scores but not CDI mean length of longest utterance and total words out of 396 and 680, PLS-4 nor SRS total raw score.	Higher age and IQ associated with more total utterances (NS effects for sex); baseline MSEL visual reception a significant predictor of total and imitative utterances. Treatment effect not modified by baseline PLS, CDI nor SRS scores.

(Continued)

TABLE 3 | Continued

Study	Participants		Intervention		Dependent Variables	Outcomes	Predictors
	PRT	Other	PRT	Other			
McDaniel et al. (58)	$N = 20$; $M_{age} = 49.85$ mths, $SD = 11.92$; 12% Female; Ethnicity: White (28%), Latino (7%), Asian (56%), Native Hawaiian (2%), Multi-racial/other (7%).	$N = 20$ $M_{age} = 46.85$ mths, $SD = 9.66$; 12% Female, Ethnicity: White (30%), Latino (5%), Asian (60%), Multi-racial (0%), Other (5%).	PRT-P: Setting: clinician delivered plus parental component; Intensive phase: Duration: 12 weeks; Intensity: 10h/pw in-home clinician delivered; 1h/pw parent training; Maintenance phase: Duration 12 weeks; Intensity: 5h/pw in-home clinician delivered; 1 h/pw parent training.	DTG	Reciprocal vocal contingency derived through an automated process from daylong audio samples from the child's natural environment.	No significant group differences at baseline and 12 weeks but PRT-P had significantly higher-ranked reciprocal vocal contingency scores at 24 weeks (moderate effect size).	NR
Mohammadzaheri et al. (59)	$N = 15$; $M_{age} = 110.67$ mths, $SD = 18.71$; 40% Female; Ethnicity: Iranian (100%).	$N = 15$; $M_{age} = 110.47$ mths, $SD = 18.62$; 40% Female; Ethnicity: Iranian (100%)	PRT Setting: clinician delivered Duration: 3 months; Intensity: 60 min per session (child-clinician, parents not present), 2 sessions/pw.	ABA: Setting: clinician delivered Duration: 3 months; Intensity: 60 min per session (child-clinician, parents not present), 2 sessions/pw.	MLU; CCC.	PRT group significantly higher MLU and CCC gains than ABA group	NR
Mohammadzaheri et al. (33)	$N = 15$; $M_{age} = 110.67$ mths, $SD = 18.71$; 40% Female; Ethnicity: Iranian (100%).	$N = 15$; $M_{age} = 110.47$ mths, $SD = 18.62$; 40% Female; Ethnicity: Iranian (100%)	PRT Setting: clinician delivered Duration: 3 months; Intensity: 60 min per session (child-clinician, parents not present), 2 sessions/pw.	ABA: Setting: clinician delivered Duration: 3 months; Intensity: 60 min per session (child-clinician, parents not present), 2 sessions/pw.	Disruptive behavior (defined as any behavior that disrupted the session) coded from the videotaped fist and last session (first, middle and last 8 min).	At baseline, PRT group had a significantly higher level of disruptive behaviors; both groups showed a significant decrease in disruptive behaviors with the magnitude of reduction more pronounced in PRT than ABA group (9.9 vs. 1.2 min).	NR
Nefdt et al. (60)	$N = 13$; $M_{age} = 38.92$ mths, $SD = 14.57$; Ethnicity: not reported in detail, 81% white across both PRT and control group.	$N = 14$; $M_{age} = 38.43$ mths, $SD = 11.20$.	PRT: Self-directed learning program consisting of education material (DVD lasting 1 h 6 min and manual).	Waitlist	Parental measures: (i) Fidelity of implementation (the following five points were scored: presenting clear opportunities, child choice,	PRT group had significantly higher scores across all dependent variables at posttest that the waitlist group; All parents who completed the self-directed learning program reported high ratings of satisfaction.	NR

(Continued)

TABLE 3 | Continued

Study	Participants		Intervention		Dependent Variables	Outcomes	Predictors
	PRT	Other	PRT	Other			
					immediate contingent consequences, natural reinforces, reinforcing verbal attempts and correct verbal responses); (ii) Language opportunities; (iii) Observed parental confidence Child measures: Functional verbal utterances		
Schreibman and Stahmer (53)	$N = 20$; $M_{age} =$ 29.5 mths, $SD = 6.9$ 10% Female; Ethnicity: not reported.	$N = 19$; $M_{age} = 28.9$ mths $SD = 4.2$; 15.8% Female; Ethnicity: not reported.	PRT used by parents and therapists to target the development and spontaneous use of functional spoken language. For the first 15 weeks, there were biweekly, 2h parent education sessions (with their child) in the laboratory and additional 2 h/pw child sessions in the home (trained undergraduate student therapists); Additional 8 weeks consisted of five 2 h/pw parent educations sessions and two 2 h/pw in the home with the child.	PECS used by parents and therapists to teach children to use picture icons to communicate; For the first 15 weeks, there were biweekly, 2h parent education sessions (with their child) in the laboratory and additional 2 h/pw child sessions in the home (trained undergraduate student therapists); Additional 8 weeks consisted of five 2 h/pw parent educations sessions and two 2 h/pw in the home with the child.	Spoken Language (MSEL Expressive language scale); Spoken Vocabulary (EOWPVT and CDI); Adaptive Communication (VABS); Parent Satisfaction.	Children in both intervention groups demonstrated increases in spoken language skills, with no significant difference between the two conditions. Seventy-eight percent of all children exited the program with more than 10 functional words; Parents were satisfied with both PRT (rating 5.7 out of 7) and PECS (rating 6 out of 7).	NR

(Continued)

TABLE 3 | Continued

Study	Participants		Intervention		Dependent Variables	Outcomes	Predictors
	PRT	Other	PRT	Other			
Vernon et al. (40)	$N = 12$; $M_{age} = 35.75$ mths, $SD = 9.31$ 8% Female; Ethnicity: White (75%), Latino (17%), Asian (8%), Multi-racial (0%).	$N = 11$; $M_{age} = 34.45$ mths, $SD = 10.08$; 18% Female; Ethnicity: White (36%), Latino (27%), Asian (18%), Multi-racial (18%).	PRISM Model: Duration: 6 mths Intensity: up to 10 h/pw (8 h clinician one-on-one; 2 h parent education); Mean intensity= 6.81h (25% families met the threshold of 80% completion of all possible treatment hours).	Waitlist	Primary: ADOS-2; MSEL Composite; PLS-5 Total; PPVT-4; EVT-3; VABS ABC score. Secondary: MSEL (Visual reception, fine motor, expressive and receptive language); PLS-5 (Auditory and expressive comprehension); VABS (Communication, daily living, socialization, motor skills).	For the treatment group, statistically significant changes from baseline were found for all the primary outcomes apart from the EVT-3 and VABS ABC; For the secondary outcomes, there were significant changes for MSEL Visual reception, fine motor and expressive but not receptive language scores, significant changes for VABS communication but not other VABS subscales, no changes for PLS-5 subscales were found. No significant changes from baseline were observed on any measures in the waitlist group for primary outcomes. For secondary outcomes, significant pre-post changes were observed in the Mullen scale of fine motor skills.	NR

ADOS, Autism Diagnostic Observation Schedule; BOSCC, Brief Observation of Social Communication Change; CDI, MacArthur-Bates Communicative Development Inventories; CGI, Clinical global impression; DTG, Delayed treatment group; EOWPVT, One-Word Picture Vocabulary Test; MLU, Mean length of utterance; MSEL, Mullen Scales of Early Learning; NR, not reported; NS, Not significant; NVIQ, Non-verbal intelligence quotient; PECS, Picture Exchange Communication System; PEG, Psychoeducation group; PLS, Preschool Language Scale; PRISM, Pivotal Response Intervention for Social Motivation; PRT, Pivotal Response Training; SC, social and communication; SI, social interaction; SLO, Structured language observation; SMD, standardized mean difference; SRS, Social Responsiveness Scale; VABS, Vineland Adaptive Behavior Scales.

TABLE 4 | Comparison of treatment effectiveness between pivotal response treatment and control groups.

	Difference of means	SE	*t*	*p*
SLO	0.39	0.17	2.01	0.09
CDI	0.06	0.21	0.27	0.81
VABS daily living	−0.04	0.25	−0.16	88
VABS expressive	0.41	0.25	1.62	0.26
VABS receptive	0.08	0.84	0.09	0.93
VABS socialization	−0.04	0.28	−0.15	0.89
MSEL expressive	0.03	0.20	0.14	0.89
MSEL receptive	0.05	0.21	0.25	0.81
MSEL composite	0.11	0.25	0.44	0.70
PLS-5 expressive	2.08	2.96	0.70	0.52
SRS-2 total score	−8.09	4.91	−1.64	0.24

CDI, MacArthur-Bates Communicative Development Inventories; MSEL, Mullen scales of early learning; PLS-5, Preschool Language Scale, Fifth Edition; SLO, Structured Language Observation; VABS: Vineland Adaptive Behavior Scales.

to provide important additional insights into the effectiveness of PRT.

Meta-Analytic Review

Ten published RCTs were identified (33, 38–40, 53, 56–60). Importantly, several papers reported data from the same study subjects. Specifically, the two papers by Mohammadzaheri et al. (33, 59) were based on the same sample. The study by Barret et al. (56) reported data from the same subjects as Vernon et al. (40). Finally, the McDaniel et al. (58) paper analyzed the same subjects from the Gengoux et al. (39) RCT. The study by Nefdt et al. (60) was of very low intensity and involved only instructional video material that lasted 1 h and 6 min, therefore, findings were only narratively summarized and were not included in the meta-analysis.

Detailed information on participant and intervention characteristics, dependent variables, outcomes and predictors are provided in **Table 3**. **Table 4** provides a summary of the comparisons between PRT and control groups.

Participant Characteristics

Studies included 130 (sample size range 12–25) participants receiving PRT and 122 (sample size range 11–23) children in the control groups. Children's age ranged from 1.5 to 6 years except for Mohammadzaheri et al. (59) and Mohammadzaheri et al. (33) and de Korte et al. (57) who included children older than 6. The percentage of female participants ranged between 8% (40) and 40% (33, 59). No studies provided information on parental and clinician/staff characteristics.

Intervention Characteristics

Three publications compared PRT to waitlist group (40, 56, 60), two to traditional ABA (33, 59), three to treatment as usual (39, 57, 58), one to parent psychoeducational program (38), and one to the Picture Exchange Communication System (53). Intervention duration varied widely, from one session (60) to 6 months (39, 40, 56). Similarly, intervention intensity varied including 1.5 h in total (60), 2 h of parent education sessions (with their child) in the laboratory, 2 h child sessions in the home per week (53), and a combined weekly parent training session and in-home clinician delivered therapy for 10 h per week for the first 3 months and 5 h during the second 3 months (39).

Dependent Variables

Eight studies focused on language and communication primary outcomes, utilizing observational coding (38, 39, 56, 59, 60), questionnaire measures such as MacArthur-Bates Communicative Development Inventories (38, 39, 53) and clinician-administered tests such as Peabody Picture Vocabulary Test or Mullen's Scales of Early Learning (39, 40, 53). Only one study (58) utilized an automated coding protocol to assess vocal reciprocity. Four studies assessed social interaction using direct observation (39, 40, 56), clinical global impression scale (38, 39) and parent-report measures such as the Social Responsive Scale (SRS-2) (38, 39, 61). Three studies reported outcomes for adaptive functioning (39, 40, 57). Two studies reported outcomes for cognitive functioning (39, 40) and disruptive behaviors (33, 57), each. Only one study reported effects on parental well-being (57).

Intervention Outcomes

Communication

Figure 2 shows synthesized evidence across a range of communication measures. There was evidence of statistically significant increase from baseline to follow-up in PRT group for structured laboratory observation (SLO) (4 studies; SMD:0.45, 95% CI: 0.21; 0.69), CDI [number out of 680 words CDI score from Gengoux et al. (39) and Hardan et al. (38) and raw number of words from Schreibman and Stahmer (53) were combined] (3 studies; SMD:0.45, 95% CI: 0.16; 0.74), Mullen Scales of Early Learning (MSEL) Expressive (3 studies; SMD:0.31, 95% CI: 0.04; 0.58), MSEL Receptive (3 studies; SMD:0.51, 95% CI: 0.23; 0.80) and VABS Expressive raw score (2 studies; SMD: 0.55, 95% CI: 0.16; 0.95) variables, but not for VABS Receptive raw score (2 studies; SMD:0.90, 95% CI: −0.47; 2.27) and Preschool Language Scale (PLS-5) Expressive score (3 studies; SMD: 1.37, 95% CI: −2.53; 5.27). Wide CI and the heterogeneity prevent strong conclusions with regards to VABS Expressive (I^2= 39.8%), Receptive (I^2= 93.0%) and to a lesser degree CDI (I^2= 24.6%) dependent variables. There were no significant differences between PRT and control treatment groups on any of the treatment outcomes (**Table 4**). However, the standardized mean change effect estimate for the baseline/follow-up change was higher for the PRT treatment group than the control treatment group (**Table 4**).

Several studies reported communication-related outcomes that could not be included in the meta-analysis due to non-overlapping measures. Outcome measures ranged from objective assessments such as automated process to derive reciprocal vocal contingency from daylong audio samples from the child's natural environment (58) to parent reports of different aspects of communication such as the Child Communication Checklist and the Preschool Language Scales [e.g., (40, 60)]. Interestingly,

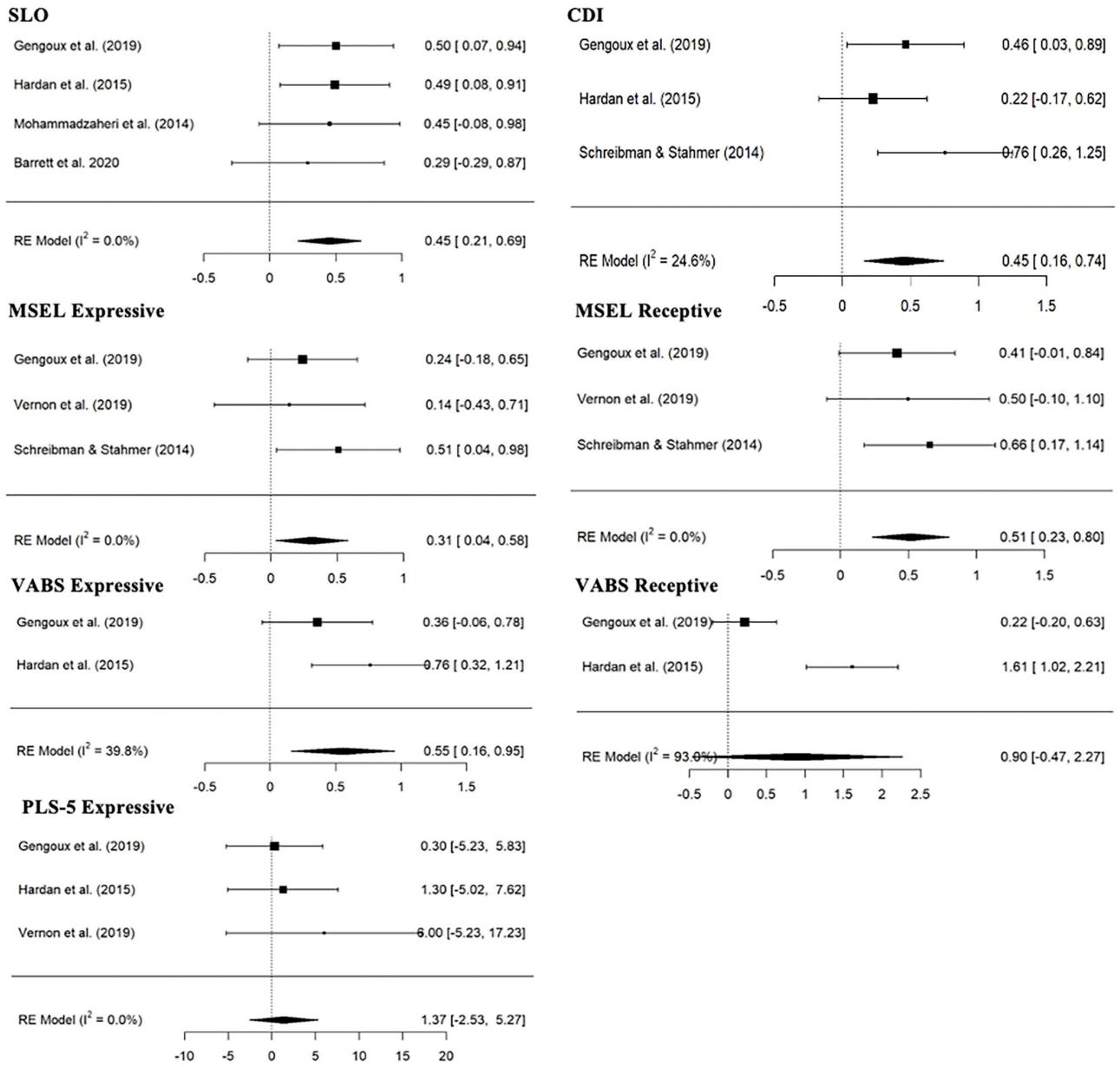

FIGURE 2 | Forest plot of estimates for PRT effects across communication measures. CDI, MacArthur-Bates Communicative Development Inventories; MSEL, Mullen Scales of Early Learning; PLS-5, Preschool Language Scale; SLO, structured laboratory observation; VABS, Vineland Adaptive Behavior Scales.

positive effects were reported on the majority of these measures (**Table 3**).

Social Interaction

Given the non-overlapping assessments utilized across the studies, it was possible to combine only VABS Socialization Standard scores for the meta-analysis. As can be seen from **Figure 3**, there was no evidence of positive effects of PRT (SMD: 0.10, 95% CI: −0.16; 0.37) in the PRT group, and no significant differences between PRT and control group were found (**Table 4**). Positive treatment effects were reported on CGI improvement social communication subscale (38, 39), the social subscale of the Brief Observation of Social Communication Change (BOSCC) (39) and child social responsiveness coded from parent-child play interaction (56) but not on the SRS-2 social communication raw score (39).

Adaptive Functioning and Cognitive Ability

Meta-analysis indicated no significant PRT treatment effects for VABS Daily Living skills subscale standard score (**Figure 4**; 2 studies; SMD: 0.31, 95% CI: −0.03; 0.65) nor MSEL Composite (**Figure 5**; 3 studies; SMD: 0.15, 95% CI: −0.17; 0.48). Wide CI

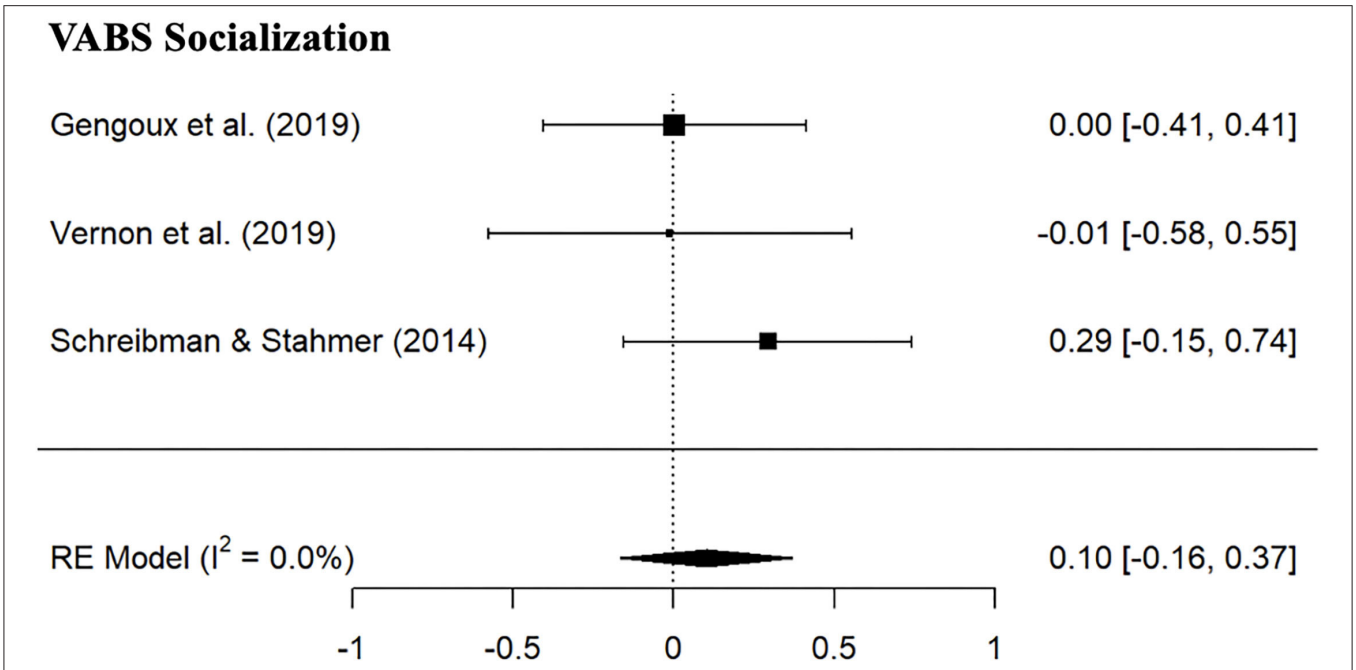

FIGURE 3 | Forest plot of estimates for PRT effects for Vineland Socialization Standard scores.

and the heterogeneity prevent strong conclusions with regards to MSEL Composite (I^2= 30.4%). There were no significant differences between the PRT and the control group (**Table 4**).

ASD Symptomatology

Meta-analysis indicated no significant PRT treatment effects for SRS-2 Total score (**Figure 6**; 2 studies; SMD: −6.03, 95% CI: −13.45; 1.40). Wide CI prevents strong conclusions. There were no significant differences between the PRT and the control group (**Table 4**). Individual studies indicated significant treatment effect on objective indexes of ASD symptom severity such as total BOSCC (39) and the total Calibrated Severity Score of the Autism Diagnostic Observation Schedule (40) (**Table 3**).

Maladaptive Behaviors

Two studies focused on exploring the treatment effects of PRT for the reduction of disruptive behaviors (33, 57). Mohammadzaheri et al. (33) reported that both PRT and ABA groups showed a significant decrease in disruptive behaviors, however, the magnitude of reduction was more pronounced in PRT than ABA group (length of disruptive behaviors reduction was 9.9 min in PRT and 1.2 min in ABA group). Of note, the PRT group had a significantly higher level of disruptive behaviors at baseline. De Korte et al. (57) reported significant reduction in behavioral problems as measured by the Brief Problem Monitor-Parents total score in PRT group.

Parental Outcomes

Two studies assessed parental satisfaction with the intervention program. Both Schreibman and Stahmer (53) and Nefdt et al. (60) reported high ratings of satisfaction with PRT (and PECS in the case of Schreibman and Stahmer). Only one study explored effects on parental stress (57) and found no evidence for improvement in this outcome as measure by the Parenting Stress Questionnaire.

Treatment Fidelity

All studies reported treatment fidelity with the exception of Mohammadzaheri et al. (33). However, this investigation is based on a similar trial by the same group where fidelity figures were included (59). Across all studies, ≥80% of parents and clinicians reached fidelity at the end of the trial. However, studies did not explicitly report steps taken if interventionists did not meet the standard.

Publication Bias

Egger's test revealed no significant ($p < 0.05$) publication bias in any of the language measures. Furthermore, visual inspection of the funnel plots did not indicate any evidence of asymmetry.

Outcome Predictors

There was not enough power to conduct the meta-regression. Several individual studies explored predictors of treatment response. Barrett et al. (56) noted that a minimally verbal subgroup ($N = 5$) showed large effect sizes for all pre- to post-treatment comparisons (child social responsiveness, number of total and different words), but the difference did not reach significance when compared to the verbal subgroup. In addition, within the minimally verbal group, initial rates of

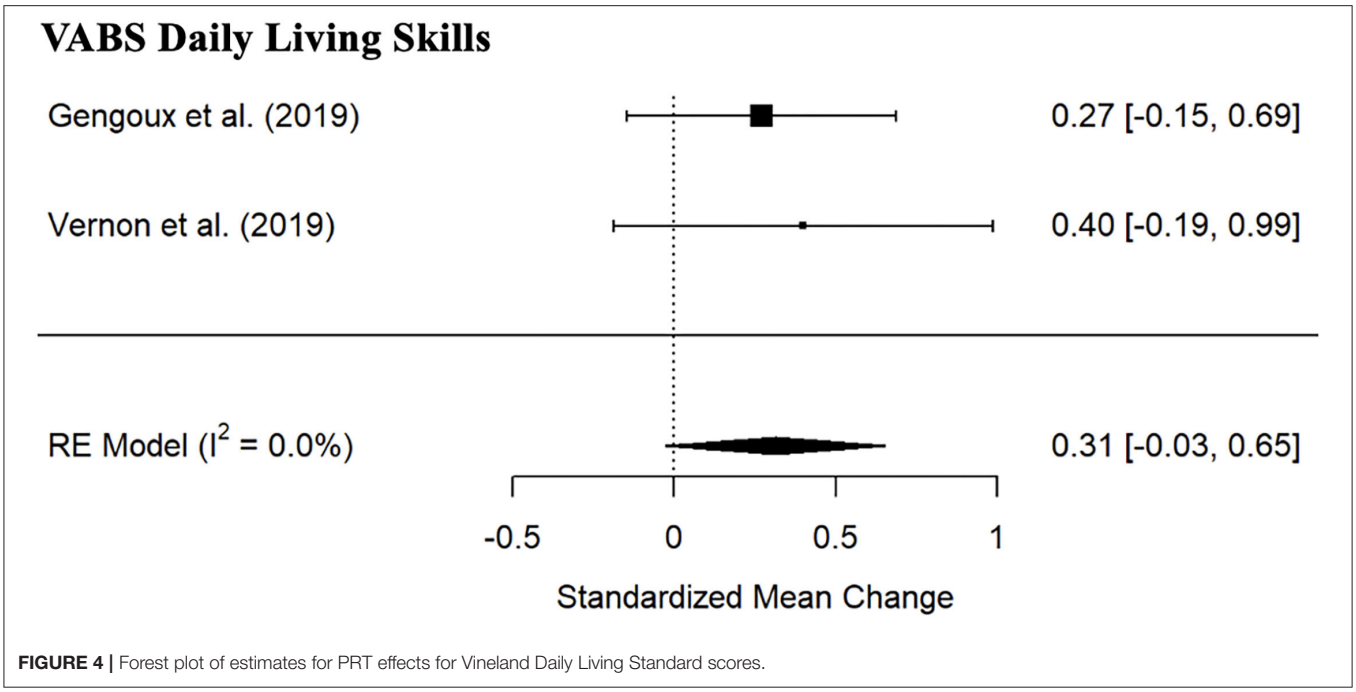

FIGURE 4 | Forest plot of estimates for PRT effects for Vineland Daily Living Standard scores.

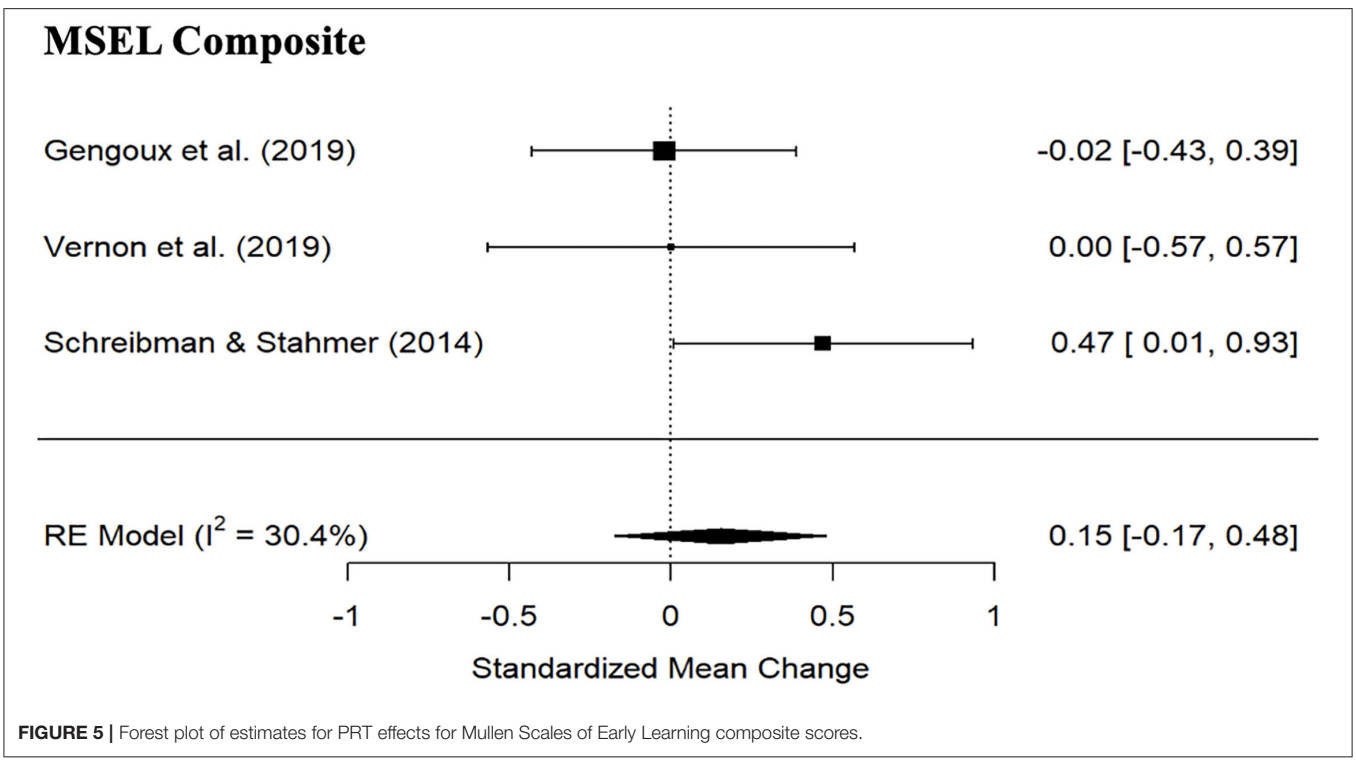

FIGURE 5 | Forest plot of estimates for PRT effects for Mullen Scales of Early Learning composite scores.

child responsiveness were strongly associated with subsequent gains in total words and moderately, but not significantly, associated with gains in different words and mean length of utterance; no significant associations were identified at the whole group level. Gengoux et al. (39) found that age, sex, and other baseline characteristics including developmental level (MSEL score) did not predict changes in treatment effect on any of the outcomes, however, the lower MSEL score, in particular the non-verbal subscales, were significantly associated with greater improvement on the BOSCC total score. Hardan et al. (38) found that while higher baseline MSEL visual reception scores were a significant predictor of treatment response for total and

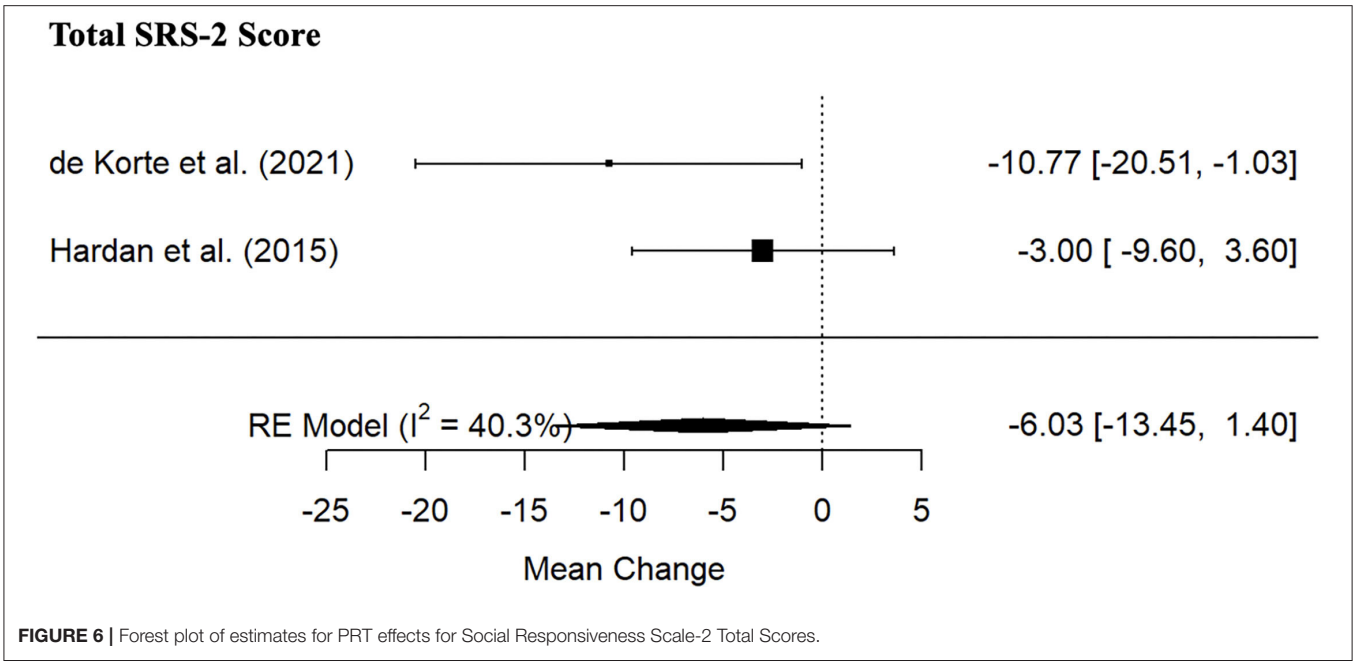

FIGURE 6 | Forest plot of estimates for PRT effects for Social Responsiveness Scale-2 Total Scores.

imitative utterances, the treatment effect was not modified by sex nor baseline PLS, CDI nor SRS scores. De Korte et al. (57) found no significant correlations between age, sex and IQ with SRS outcomes, however, they reported that lower symptom severity on ADOS CSS total score associated with higher improvements in the SRS-2 scores in PRT (but not TAU) group.

Quality Indicators

Study level quality indicators are presented in **Supplementary Table 2**. There was incomplete/insufficient information to ascertain (i) randomization in three studies (33, 59, 60), (ii) blinding in two studies (40, 56), (iii) attrition in one study (60). Effect size-specific measurement quality indicators are presented in **Supplementary Table 3**. Majority of studies utilized distal measures and generalized contexts with low correlated measurement error bias.

DISCUSSION

The current study aimed to provide a comprehensive appraisal of the current evidence on the effectiveness of Pivotal Response Treatment (PRT) for individuals with ASD through an umbrella review of previous systematic examination of the literature and meta-analytic synthesis of all available randomized controlled trials (RCT) of PRT. Overall, both previous systematic work and a new meta-analysis of the RCTs suggest that PRT shows promise for improving language and communication. However, evidence for improvements in other areas is less strong. Crucially, only three studies examined predictors of intervention outcomes.

Our umbrella review captured six systematic examinations of the literature specifically focusing on PRT, two with a meta-analytic component and four providing a descriptive summary of the findings. These reviews varied widely in terms of their aims, outcome, and designs. One of these studies aimed at appraising treatment effectiveness (37) while another focused on adherence to specific research quality standards (48). One review aimed to capture comprehensive outcomes across a number of domains (37) while another one targeted communication only (49). Reviews captured different designs with one focusing on single case reports (28), one on RCTs (46), and another on a combination (37). Therefore, it is difficult to form a unified and consistent set of conclusions and recommendations. However, several observations can be made. The majority of the reviews encompassing all study designs provided evidence that PRT was effective for certain aspects of language and communication (28, 37, 46, 49). Importantly, the positive effects of PRT were observed across assessment methods. The only exception was a systematic review by Boudreau et al. (47) that concluded that, based on the criteria put forward by Reichow et al. (50), none of the included studies could be classified as promising or established for improving social-communication impairments. However, Boudreau et al. (47) included 5 single case studies (with 10 participants in total) that focused only on peer-mediated PRT which may explain the lack of significant treatment effects. Other outcomes (e.g., adaptive functioning, cognitive functioning, overall ASD severity) were less frequently appraised and therefore it is difficult to ascertain evidence of PRT effectiveness for non-language/communication outcomes. Additionally, each review raised a range of limitations of the identified studies that can be systematized into the following three broad categories. The first is related to the nature and comprehensiveness of appraised outcomes and the type of assessments, in particular the need to incorporate more objective measures and capture parental outcomes. The second category included the lack of understanding of predictors of response and active treatment ingredients. Finally, the third group is related

to the lack of understanding of parental and staff predictors of effective treatment implementation.

Eight of ten identified RCTs reported at least one language and communication-related outcome and it was possible to conduct six meta-analyses across different measures with a number of synthesized studies varying between two (for VABS expressive and receptive subscale), three (for CDI, MSEL expressive and receptive subscale, and PLS-5 expressive subscale) and four (SLO for assessment of utterances). Our meta-analysis indicated clear benefits in language abilities from PRT. A statistically significant increase from baseline to follow-up in the PRT group was observed for both objective (SLO, MSEL expressive and receptive scores) and parent- and/or clinician-report (CDI, VABS expressive score) measures of language and communication. However, no differences from baseline were observed on the VABS receptive scale and the PLS-5 expressive scale. A range of other language and communication outcomes that could not be synthesized in the meta-analysis also indicated positive treatment effects. These encompassed positive effects on both the parent- and/or clinician-reports including the Children's Communication Checklist (CCC) (59), the One-Word Picture Vocabulary Test (EOWPVT) (53), the Peabody Picture Vocabulary Test (PPVT-4) (40) and automatic coding of vocal reciprocity (58). The only exception was the Expressive Vocabulary Test (EVT-2) total score that did not significantly improve as a result of PRT (40).

PRT studies have also examined a range of non-language target behaviors. Five studies to date used outcome measures to assess overall ASD severity, adaptive functioning, cognitive functioning, and disruptive behaviors. Only one meta-analysis, although limited by the number of studies, was possible for social interaction (2 studies, VABS Socialization scale), overall autism symptom severity (2 studies, SRS-2 Total Score), adaptive functioning (2 studies, VABS Daily Living scale) and cognitive functioning (3 studies, MSEL Composite) each, indicating no significant PRT treatment effects for these outcomes. A wide CI and considerable heterogeneity prevent strong conclusions regarding cognitive functioning. It is also important to highlight that results from individual studies that were not possible to be synthesized in meta-analysis suggested significant treatment-related improvements for social interaction measured by CGI (38, 39), BOSCC social subscale (39) and parent-child interaction coded for social responsiveness (58), but no effect on the SRS-2 Social Communication and Interaction raw score (39). Similar improvements related to overall ASD symptoms were observed as measured by the BOSCC total score (39) and Autism Diagnostic Observation Schedule (ADOS) Calibrated Severity Score (CSS) total score (40), however, meta-analysis based on two studies (38, 57) indicated no significant positive effects for the SRS-2 total score (38). Finally, positive treatment effects of PRT were also reported in reducing the magnitude of disruptive behaviors (33, 57).

Only four studies examined predictors of treatment outcomes. Although not significant, the findings reported by Barrett et al. (56) provide some evidence that a minimally verbal subgroup ($N = 5$) might show better treatment response compared to the verbal subgroup. Further, Barrett et al. reported that, while initial rates of child responsiveness were not predictive of subsequent outcomes at the group level, they were associated with subsequent gains of vocabulary in the minimally verbal group. Hardan et al. (38), De Korte et al. (57), Gengoux et al. (39) found that age and sex were not related to subsequent outcomes; however, they reported inconsistent findings with regards to the effects of IQ. More specifically, while Gengoux et al. (39) found that the lower MSEL score, in particular the non-verbal scores, were significantly associated with greater improvement on the BOSCC total score, Hardan et al. (38) found that higher baseline MSEL visual reception scores were a significant predictor of treatment response for total and imitative utterances and de Korte et al. (57) reported no significant associations between IQ and outcomes. De Korte et al. (57) reported that lower severity of autism symptoms at the baseline was associated with higher improvements in the SRS-2 scores in PRT group. Therefore, based on the currently existing evidence, it is not possible to identify a consistent pattern of baseline characteristics that are associated with PRT treatment outcomes.

Limitations and Future Directions

Despite a notable increase in the number of PRT RCTs in the last few years, identified studies were all limited by small to moderate sample size, a significant limitation that needs to be taken into account when appraising the current body of evidence for the effectiveness of PRT. When interpreting the comparisons of the effectiveness between PRT and control groups, it is important to note that due to the limited number of RCTs identified, it was not possible to conduct separate analyses for RCTs that used active (e.g., ABA) and waitlist control groups. In addition, this systematic review has identified several other key limitations that should be addressed in future research. Firstly, future studies will need to include more comprehensive treatment targets, in particular adaptive functioning, a generalization of treatment effects and longer-term (12-months or longer) outcomes. In addition, only one of the identified RCTs have explored the effects of PRT on parental well-being, reporting no significant beneficial effects on parental stress (57). Comprehensive understanding of the effects of PRT on parents, both direct and indirect, is particularly crucial given that high levels of stress, anxiety and depression and poorer quality of life among parents of children with ASD are well established (62–66). It is encouraging that several RCTs have shown positive treatment effects on objective measures, therefore reducing the risk of bias, however, this approach should become a standard practice for future studies. Additionally, it is well recognized that a range of currently available, standardized ASD diagnostic and quantitative severity measures such as the Autism Diagnostic Interview-Revised (ADI-R), the ADOS and the SRS-2, have limited sensitivity to change and response to interventions (67) which restricts their utility in the context of clinical trials. Recently, the Brief Observation of Social Communication Change (BOSCC) has been developed as a measure for capturing the change of core ASD symptoms. Despite promising initial findings (68–70), the BOSCC RRB domain appears to be less sensitive to changes (39). Therefore, further development of instruments able to capture

the subtle change in distinct symptom domains is an area of urgent need.

Individual differences in treatment response among individuals with ASD are well established (71–73). Although baseline characteristics such as gross measures of cognitive and language level and overall ASD severity have been found to predict response across a range of existing treatments (74, 75), our field lacks a comprehensive understanding of specific factors underlying individual variability in response to particular intervention and treatment components and is therefore missing crucial information for enabling individualization of treatments (76, 77). One of the major benefits of well-powered RCTs is the ability to characterize predictors of treatment response and how and why specific interventions benefit individuals with ASD (41). However, although four identified PRT RCTs have explored predictors of treatment outcomes (38, 39, 56, 57), a combination of sample size, analytic and methodological limitations did not allow us to conduct meta-regression and gain more robust insights into specific predictors of PRT response. Therefore, it will be crucial for future PRT RCTs to improve trial methodology by adopting factorial designs, comparative efficacy trials and adaptive treatment designs while implementing more advanced individual difference analytical strategies that would enable the identification of subgroups of children who respond well to PRT and understand the profile of treatment responders.

CONCLUSIONS

Statistically significant effects of PRT on a range of language and communication skills were identified across a majority of ten RCTs included in this review. This finding is in line with the hypothesis that increasing social motivation and thus the quality and quantity of opportunities for social learning will yield positive downstream effects on language and communication abilities (21). However, evidence for positive treatment effects of PRT on outcome measures assessing other domains was less robust and specific. This review has identified that several key methodological and design improvements are needed to enable our field to fully leverage the potential of RCT designs and establish not only overall treatment efficacy but, more importantly, detailed profiles of treatment responders and therefore provide evidence-based guidance for clinicians on what works for whom and why.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

MU and AH designed the study. PC and MU conducted the systematic search. MU and AH screened the eligible studies and extracted the data. WB, MC, and MU conducted the analyses. MU, AH, WB, and PC drafted the initial manuscript. All authors critically reviewed, provided feedback on the initial version of the manuscript, and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpsyt.2021.766150/full#supplementary-material

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Rethinking Autism Intervention Science: A Dynamic Perspective

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Recent advances in longitudinal methodologies for observational studies have contributed to a better understanding of Autism as a neurodevelopmental condition characterized by within-person and between-person variability over time across behavioral domains. However, this finer-grained approach to the study of developmental variability has yet to be applied to Autism intervention science. The widely adopted experimental designs in the field—randomized control trials and quasi-experimental designs—hold value for inferring treatment effects; at the same time, they are limited in elucidating *what* works for *whom*, *why*, and *when*, given the idiosyncrasies of neurodevelopmental disorders where predictors and outcomes are often dynamic in nature. This perspective paper aims to serve as a primer for Autism intervention scientists to rethink the way we approach predictors of treatment response and treatment-related change using a dynamic lens. We discuss several empirical gaps, and potential methodological challenges and opportunities pertaining to: (1) capturing finer-grained treatment effects in specific behavioral domains as indexed by micro-level within-person changes during and beyond intervention; and (2) examining and modeling dynamic prediction of treatment response. Addressing these issues can contribute to enhanced study designs and methodologies that generate evidence to inform the development of more personalized interventions and stepped care approaches for individuals on the heterogeneous spectrum of Autism with changing needs across development.

Keywords: autism (ASD), intervention outcome, developmental trajectories, time-varying (TV), longitudinal, prediction

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INTRODUCTION

Over the past decade, the field of Autism intervention science has made significant advances that led to promising evidence on improving the developmental outcomes of individuals with Autism (1). However, methodological concerns such as small sample size, detection bias related to limited informant types and objective outcome measures, and restricted trial contexts, continue to limit the replicability and generalizability of these findings (1–3). Recent meta-analytic studies revealed empirical gaps in the prediction of differential treatment response and mechanisms through which treatments work, potentially due to limited statistical power and discrepancies in designs and reporting practices across studies (1, 4).

Certain conceptual limitations in manipulating and evaluating treatment-related change could also be a barrier to advancing personalized care in Autism. Specifically, Autism intervention science has historically relied on traditional randomized control trials (RCTs) and quasi-experimental methodologies that often do not account for the heterogeneous and dynamic nature of Autism.

While being useful in yielding causal inferences of treatment effects, RCTs evaluating Autism interventions use the process of randomization to "control" statistically for the possible influence of static "confounding" factors at baseline not under direct experimental control. Also, treatment response in RCTs is often determined by comparing group (experimental vs. control) differences or subtracting placebo response from the overall response, thus being limited in assessing individual-level treatment response (5). This is particularly relevant to Autism, as we know from observational longitudinal studies that variable developmental trajectories can be identified among autistic individuals over the life span in several behavioral domains that are often the targets of Autism intervention studies, such as core symptoms of Autism, adaptive functioning, IQ, and challenging behavior (6–9). The waxing and waning of target outcomes across development may contribute to the variable treatment response, but it is difficult to differentiate the sources of variability under the traditional experimental designs. For instance, some target treatment outcomes may decrease over a longer time span as individuals grow out of certain behaviors (e.g., from non-verbal to verbal communication), resulting in an artifact of reduced treatment response (10). Although the traditional RCT design has proven to be invaluable and thought of as the gold standard of evidence for the study of other—mostly physical—disorders, the derived findings are often limited in generalization beyond the trial sample given the restrictions mentioned above (11). Considering the idiosyncrasies of neurodevelopmental disorders where behavioral manifestations are often dynamic and heterogeneous in nature, there is a need to rethink how we approach the study of Autism intervention ingredients, including both predictors and outcomes, to better elucidate *what* works for *whom*, *why*, and *when*.

CAPTURING INDIVIDUAL-LEVEL VARIABILITY IN TREATMENT OUTCOMES

In classical RCTs and quasi-experimental designs, it is common to collect outcome data at pre- and post-treatment, sometimes with post-treatment follow-up. While this satisfies the purpose of inferring whether the treatment is more effective than placebo, the "black box" of what happens *during* treatment remains unopened (see **Figure 1**). Further, the treatment-related change is often treated as a "chunk" averaged across individuals (e.g., average treatment effect, average effects on the treated) rather than a continuous process over time (e.g., within-person change) for each individual. While strategies such as subgroup analysis and propensity scores based on a priori groupings (e.g., sex) can be used for addressing heterogeneous treatment effects (12), it could still be problematic when observations do not correspond to individual experience or behavior in a non-ergodic (i.e., non-stationary and variable) behavioral change process in the real world, thus limiting the generalization and replication of the findings (13, 14). It also poses challenges in differentiating between individual treatment response and random variability that may bias the evaluation of treatment response. Some potential sources of bias include natural fluctuations of treatment outcomes, response bias (e.g., tendency to report favorable outcomes), practice effects, statistical artifacts (e.g., regression to the mean, ceiling/floor effect) (15). Finally, although randomization helps to increase the internal validity of group comparisons by making the factors associated with unobserved uncertainty equitably distributed across the treatment and the control groups, meaningful individual variability might also be distributed across the two groups. When sample size is small and/or individual variability is not well addressed with appropriate analytical approaches (e.g., accounting for within-group variations), there could be a higher probability of type II errors and thus reduced power to detect effects (16).

Although recent advances in longitudinal designs and analyses for observational studies have contributed to a better understanding of the heterogeneity of progression of Autism-related phenotypes both *within* and *between* individuals and over time—i.e., chronogeneity (17, 18)—how treatment outcomes are approached in Autism intervention research remains limited in addressing individual-level variability with respect to time. To date, there are only a handful of larger-scale intervention studies that describe developmental trajectories of intervention outcomes using approaches accounting for both within-person and between-person differences, such as multilevel modeling and latent growth curve analysis. In an RCT study (10), variable trajectories of joint attention behaviors were observed among a group of preschool-aged children diagnosed with Autism over the course of social communication intervention and 5-year follow-up, where the change patterns were associated with treatment assignment and diagnostic status at the exit. A recent observational study (19) reported an overall increase across diverse language trajectories between the entry and exit of an early intensive behavioral intervention (EIBI) program among preschool-aged children with Autism, with steeper improvements predicted by younger age, higher cognitive abilities, and lower symptom severity at baseline. Another observational study (20) examined the growth curve of autistic children's developmental outcomes across several time-points during applied behavior analysis (ABA) intervention and found that symptom severity, primary language spoken at home, and child's sex, but not treatment intensity and age of entry, were significant predictors of growth rates in certain outcomes during the intervention. Along with another observational EIBI study for children with Autism (21), different rates of improvement in treatment outcomes were observed across time-points during the period of intervention. For instance, many children tended to show patterns of exponential negative growth (faster improvement in the beginning followed by decelerated progress after). These findings suggest that treatment-related change is developmentally variable, and differences in baseline characteristics within treatment groups can be associated with various treatment responses or lead to different treatment outcomes. Also, the rate of change may vary during and beyond the intervention, potentially in a non-linear trend that is often hard to observe with limited data points. This indicates that there may be a time window for certain groups of people to better respond to the treatment.

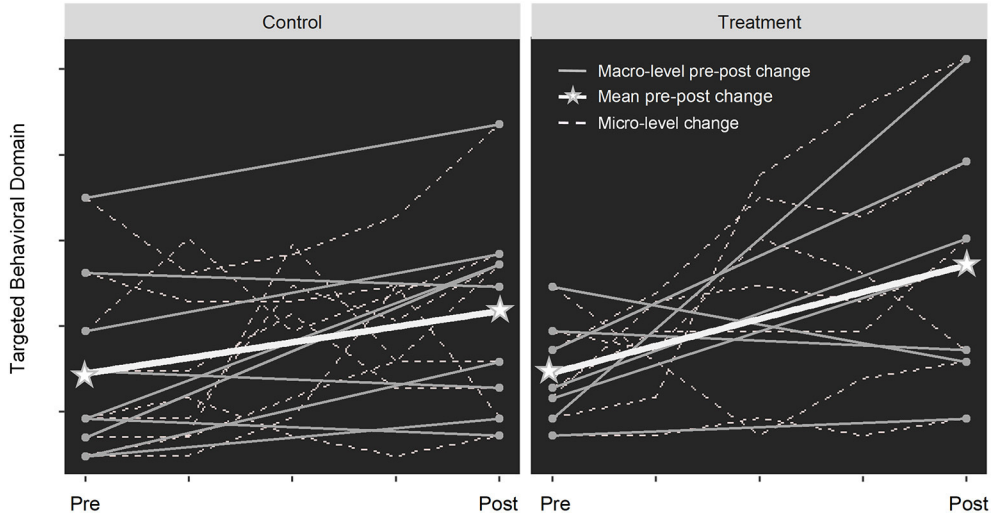

FIGURE 1 | The "black box" of treatment effect in RCTs. In an RCT design, participants are randomized to treatment or control groups with matched baseline characteristics. When only calculating the average change from pre- to post-treatment (thick white lines), the larger increase observed in the treatment group may lead to the conclusion that the treatment is effective (assuming significant group difference), despite the individual-level heterogeneous response (gray lines). When breaking the treatment period into smaller intervals, the micro-level change (dashed lines) reveals that the rate of change varies across individuals over time, indicating time-varying treatment effects. Regarding "opening the black box", we are not referring to unblinding the clinical trial procedures, but rather adopting study designs (e.g., more frequent data collection with more refined outcome measures over a longer time span) and analytical approaches (e.g., trajectory analyses) that allow for examining the finer-grained changes in treatment response.

Despite emerging evidence on the complex patterns of treatment-related change, more research that captures fine-grained variability over time is needed for informing personalized intervention in Autism. The fundamental issue might lie in the imbalance between simplicity and complexity when approaching treatment-related change with a lack of respect to the role of time in the risk and resilience process (22), thus limiting the field from yielding robust, meaningful, and translatable findings. Below we discuss some empirical gaps, methodological challenges, and opportunities that could be drawn from other fields, as well as an illustrative example for autism researchers to plan for "next steps".

Research Questions/Hypotheses

While Autism intervention science often poses the question of *what* works for *whom*, and *why*/*when* the change happened, the main research question tested empirically is *whether* the change happened due to the specific treatment. Although the latter question is foundational for demonstrating the effectiveness of treatment, it may not be sufficient in providing generalizable information on applications outside the clinical-trial settings where more variability related to individual differences or time is expected. Aside from confirming *whether* the treatment works, we may also explore the overall *shape* (i.e., progression) of change in specific proximal or distal treatment outcomes, *when* the greatest amount of change or inconsistent rate of change occurs, and *how* these patterns of change are associated with certain individual characteristics. Answers to these questions would inform the development of more tailored treatment programs that are more targeted and better timed for optimal response.

Design/Measurement

These types of research questions highlight the need for refining the tracking of treatment outcomes through appropriate study designs, including:

- More frequent measurement occasions at shorter timescales to capture finer-grained behavioral change processes of treatment targets: While intensive data collection of proximal treatment targets is a common practice in EIBI, individual variability in behavioral change has rarely been addressed. Recently, intensive longitudinal (IL) methods, such as ecological momentary assessment, experience sampling, and daily diary, have been increasingly adopted in the field of psychopathology to better capture the temporal dynamics of symptoms and functions, thus allowing for better elucidation of treatment effects mechanisms (23, 24). While it remains challenging to collect longitudinal data with validated tools in behavioral research, the recent advance in remote monitoring and telehealth methods as well as the use of accelerated longitudinal design could facilitate the feasibility of more intensive behavioral data collection during clinical trials (24, 25).
- More refined and psychometrically validated behavioral constructs as treatment outcome measures: Given the multidimensional clinical features of Autism and associated challenges, it would be useful to have measures that capture *specific* domains (or sub-domains) of targeted outcomes and other key neurobehavioural constructs (e.g., specific joint attention skills instead of a general social communication composite score) at multiple time points to be able to examine the interplay of different treatment outcome domains

over time, as well as to better account for the heterotypic development (i.e., age-dependent behavioral manifestations) of outcomes during long-term follow-up.

Analysis

Future Autism intervention research may benefit from applying the learnings achieved in observational studies describing heterogeneous developmental trajectories. Specifically, analytical approaches for studying *between-person* differences in *within-person* change (e.g., latent growth modeling and multilevel modeling), and person-centered approaches for identifying homogeneous subgroups (e.g., growth mixture modeling), can contribute to better capturing individual treatment-related change over time. These approaches allow for addressing a variety of development-related complexities, such as non-linear trajectories, time-varying predictors, and interactions across multiple treatment outcome domains. They are also flexible in dealing with some common challenges in intervention studies, such as missing data and non-normally distributed measures (26). It should be noted that these approaches often require at least three time-points of panel data to estimate linear latent trajectories or linear random effects and four time-points for capturing non-linear within-person and between-person changes (27).

Illustrative Example

te Brinke et al. (28) recruited a total of 108 adolescents with elevated externalizing behavior, who were randomized to either a treatment (emotion regulation training) group or a control group. Emotion regulation strategies and externalizing problems were assessed at baseline and at two treatment phases. At each phase (spanning 3–7 weeks), self-reports of emotion regulation difficulties and aggression were collected weekly via smartphones. Aside from examining the group differences in distal treatment outcomes (i.e., emotion regulation strategies and externalizing problems), this design allowed to examine the effect of treatment manipulation (alternating the sequence of cognitive or behavioral approaches) on proximal outcomes (i.e., emotion regulation difficulties and aggression) by modeling their piecewise trajectories across individuals in the treatment group. Similarly, autism researchers may apply such design to examine the trajectories of distal treatment outcomes (e.g., expressive language) between groups as well as finer-grained with-person changes in proximal treatment outcomes (e.g., specific joint attention skills) through more intensive data collection. Such an approach would also allow for examining the potential effect of changing intervention ingredients (e.g., sequence and dosage), which would be particularly useful under an adaptive intervention design.

MODELING DYNAMIC PREDICTION OF TREATMENT RESPONSE

As we continue to advance our work on describing treatment-related change over time, it is also important to identify predictors of "more responsive" trajectories (e.g., higher rates of improvement, longer maintenance of treatment effects). As reported in the intervention studies mentioned above (19, 20), some child demographics (e.g., age, sex) and characteristics at baseline (e.g., IQ, level of symptoms) were associated with different trajectories during and/or after the intervention. What remains unclear, however, is the dynamic processes between predictors and treatment outcomes underlying these variable trajectories. Conventionally, predictors and their effects are treated as "static" based on the assumptions that, for example, cognitive and language skills do not change beyond the baseline and their effects on intervention outcomes hold constant over time. However, as demonstrated by many longitudinal studies, the outcomes and predictors of interest are often not static [e.g., IQ and symptoms of Autism; (8)] and may have dynamic associations with each other over the period of observation [e.g., language and social skills; (29)] among individuals with Autism. Moreover, major life changes, such as a transition to school and the COVID-19 pandemic, may "disrupt" children's trajectory outcomes and their associations with predictors (30, 31). Changing intervention components (e.g., types, dose, duration, intensity of treatment) may also influence such dynamics. Recently, adaptive intervention approaches (e.g., sequential multiple-assignment randomized trials, SMARTs) have represented a promising strategy to personalize Autism intervention (32), where participants are randomized into different sequences of intervention options according to their response to treatment. These "smarter" intervention approaches require "smarter" analytic methods to better address treatment-related change. And even in the case of predictors that are invariant in nature (e.g., sex), the magnitude of their predictive effect may still vary across the course of intervention and/or development [e.g., interactions between sex and age for comorbid symptoms in children with Autism; (33)]. All these complexities regarding the prediction of treatment-related change point to the need for more "time-sensitive" approaches, such as dynamic prediction modeling that allows for examining time-varying effects on treatment outcomes (see **Figure 2**).

The concept of *dynamic prediction* is not new to the field of psychopathology, which has been adopted in intervention studies for alcohol or drug addiction and affective disorders [e.g., (34, 35)] as well as in non-intervention studies such as the prediction of mental disorder onset and progression (36, 37). The idea behind the dynamic prediction is to approach psychopathology as a system rather than as a category (37) through capturing the reciprocal relation between trajectories of interest (e.g., treatment outcomes) and their etiologically and clinically relevant time-varying predictors (34). Recently, as a response to the impact of COVID-19, a dynamic clinical prediction model has been proposed to adapt to the constantly evolving healthcare system, where predictors as associated with changes in population demographics, prevalence of disease, and clinical practice paradigms are taken into account for decision-making (38, 39). From an analytic perspective, changes that arise over time (beyond experimental control) may introduce uncertainty to prediction models and result in "calibration drift" (i.e., less accurate predictive ability over time) (40). Thus, establishing prediction models with only baseline predictors may under-utilize the available information and thus limit predictive

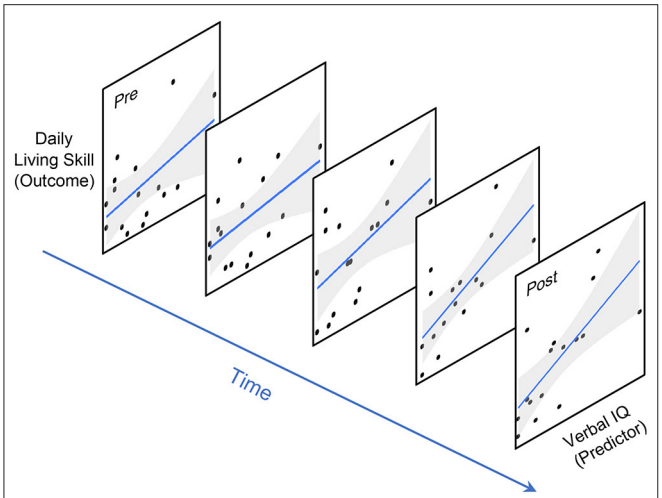

FIGURE 2 | Time-varying prediction of treatment outcome. Conventional linear regression approaches assume the effect of predictors/covariates (e.g., IQ) on treatment outcomes (e.g., adaptive skills) to be static or constant over time. In contrast, a time-varying prediction model estimates their association as a function of time.

ability and replicability (41). While our current knowledge about predictors of treatment outcomes in Autism remains inconclusive due to several conceptual and methodological limitations, such as a lack of theory-driven models with attention to individual differences (42), the missing piece of "time" may be a major factor underpowering the detection of meaningful effects.

The concept of dynamic prediction also applies to the study of treatment mediation given the nature of mediators as outcome predictors. Mediation is commonly studied with regression-based approaches in intervention studies to understand treatment processes and mechanisms. Such approaches often assume mediation effects to be linear and thus ignore that independent variable, outcome variable and mediator are typically not in a strictly unidirectional and static relation, but instead in a bidirectional relation that may change over time (42). As demonstrated in a large-scale RCT study with long-term follow-up for a parent-mediated social communication intervention targeting children with Autism (43), while the treatment effect on parental synchrony (mediator) attenuated over time, the treatment effect on child outcomes did sustain at follow-up, indicating that the mediation mechanism could vary across different stages of intervention. Such finding also supports the theoretical foundation behind development-based intervention approaches (e.g., naturalistic developmental behavioral interventions), in which developmentally-appropriate precursor skills are targeted to improve developmental outcomes. Thus, a move from static to dynamic approaches of examining predictive effects, including moderation and mediation, would not only facilitate our understanding of why and when treatment response becomes differential across individuals, but also better reflect the rationale of developmentally grounded intervention approaches.

Given the advances across the broader field of psychopathology in addressing the dynamic nature of the predictor-outcome relation, as contrasted with the common practice of studying this interplay as static in Autism intervention science, we suggest that future research may want to identify time-varying predictors or covariates of treatment outcomes based on theory and existing evidence. Here we raise some potential challenges and opportunities regarding dynamic prediction of treatment outcomes, along with an illustrative example that might be applied to Autism intervention research.

Research Questions/Hypotheses

As discussed above, some common baseline predictors, such as cognitive and language skills, may change over time and have a dynamic relation with each other and with treatment outcomes. Mediating effects could also vary across time, such as the effect of parent responsiveness on child's treatment outcomes during parent-mediated intervention vs. follow-up. In this regard, some examples of "time-sensitive" research questions that can be asked include: (1) When (e.g., 1 month upon entry, 6 months after exit) does the treatment effect become active or reduced? (2) How do individuals with certain characteristics or in different contexts (e.g., verbal vs. non-verbal, various intervention elements, levels of environmental support) differentially respond to the treatment over time (i.e., time-varying moderation of treatment outcome)? (3) Does the mediating effect on treatment outcome (e.g., parental responsiveness on child's social response) vary over time? (4) When do two or multiple behavioral domains of interest (e.g., core symptoms and comorbidities) become "decoupled" as the result of the intervention? These "time-sensitive" research questions could yield findings that fill the empirical gaps in the Autism intervention research regarding treatment timing and underlying mechanisms.

Design/Measurement

Modeling dynamic prediction of treatment response requires repeated data collections of (lagged or concurrent) outcome and predictor variables with adequate coverage across the time span. And as with any longitudinal analysis, the assumption of measurement invariance across time should be met. We note that some challenges which have hampered Autism intervention research historically, such as the burden of repeated measurements on both participants and assessors, low recruitment numbers and high attrition rates (that lead to smaller sample sizes), are still relevant here. However, lessons and opportunities could be drawn from cross-site collaborations and consortiums for genomic and biomarker data that have been developed over the past decades in the field of Autism research (44, 45) for increasing sample sizes as well as enhancing data sharing and harmonization of clinical trial data, which would allow researchers to address more complex but relevant research questions. The Autism research community needs to work together to make it possible to identify meaningful predictors of treatment response through precision approaches (3).

Analysis

As a direct extension from the widely adopted cross-lagged panel models in longitudinal studies, the incorporation of time-varying covariates could be achieved by specifying random intercept factors that represent the person-specific deviations from mean

trajectories at a specific time-point (46). A similar idea can be also applied to multivariate latent curve modeling with structured residuals that capture time-specific within-person differences in the association of multiple trajectories (e.g., the association between trajectories of treatment outcome and predictor) (47). Other novel methods which have been increasingly used in the field of psychopathology include time-varying effect modeling [TVEM; (48)], dynamic structural equation modeling [DSEM; (49)], and joint modeling (50). A shared characteristic of these methods is the non-parametric or semi-parametric estimation of regression coefficients for time-series data without a priori constraints on underlying trajectories and shapes of coefficient functions. These methods have been applied to intervention and prevention for addiction and affective disorders [e.g., (51–53)], as well as detection of transition to psychosis [e.g., (54)], and thus may be useful candidates to be applied and tested in Autism intervention science. Survival analytical approaches that are widely adopted in medical and epidemiological research, such as Cox proportional-hazards regression models, which assume log-linearity in covariates, could also be used to examine the time-varying effects of covariates (55). Finally, Bayesian approaches could be applied to handle time-varying coefficient models with greater complexity (e.g., multiple random effects) (56).

Illustrative Example

Wright et al. (51) examined the impact of co-occurring anxiety on depression treatment outcomes among 78 outpatients over the course of psychotherapy. The patients received either traditional psychotherapy for depression or a variant that also targets anxiety. Depression and anxiety symptoms were assessed by clinicians at each of the 16 weekly treatment sessions. Instead of using baseline anxiety as a predictor, the researchers examined the dynamic associations (i.e., time-specific coefficients) between anxiety and depression using TVEM to clarify whether anxiety and depression "decoupled" as treatment proceeds. They also examined whether the time-varying associations differ between groups who received different versions of treatment at certain time-points. Autism researchers could also apply a similar approach to explore research questions related to co-occurring symptoms (e.g., repetitive and restricted behaviors and anxiety) during behavioral interventions, parent-child dyads (e.g., parent responsiveness and child's joint attention skills) during parent-mediated interventions, or inclusion of predictors that may change in nature (e.g., cognitive and language skills). This would allow for elucidating how and when the covariates of interest contribute to treatment targets or interact with intervention elements at an individual level.

CONCLUSION

We discussed here two important empirical gaps in Autism intervention science that, to date, has been relying on observational or experimental designs (including RCTs) predominantly characterized by: (1) evaluating treatment effects based on group comparisons of mean pre-post changes in general outcome domains; and (2) studying prediction of treatment outcomes as a static phenomenon. The failure to measure more real-time treatment response, particularly under a pre-post design, may lead to a biased inference of treatment effects. Moreover, while it is reasonable to "control for" static predictors of intervention outcomes, the findings should be cautiously interpreted given the untested assumption that the predictors pose effects on the target outcomes that do *not* vary in strength over time. This, however, may undermine the predictive accuracy of treatment response and limit the generalization of findings to autistic populations with heterogeneous developmental profiles in real-world contexts. Given the dynamic and developmental nature of psychopathology (57), such as the gene-environment interplay that may impact developmental outcomes in Autism (58, 59), it is important to take temporal and contextual dimensions of treatment effects into account to elucidate *why* and *when* the intervention works or does not work. We discussed several methodological challenges and potential solutions for addressing these empirical gaps when designing Autism intervention studies. Adopting a dynamic lens can help researchers and clinicians to better understand the adaptive developmental processes to positive or negative changes associated with intervention or environment (59), whose importance is underscored by the pandemic's significant impacts on autistic individuals and their families (31, 60). As the field is entering the era of stepped and personalized healthcare (61), there is a need to pause, rethink, and discuss an intervention research agenda that better addresses the developmental and dynamic nature of Autism, and to adopt methodological approaches that support the shift of focus from *macro* to *micro*-level change, as well as from *static* to *dynamic* prediction of change. Such a paradigm shift would contribute to the refinement of personalized interventions tailored to heterogeneity across development (i.e., chronogeneity) so that interventions and services could be delivered to autistic individuals and their families in a timely, targeted, and adaptive manner.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

SG and Y-JC contributed to the conception of this manuscript. Y-JC drafted the manuscript with comments and edits from ED and SG. The final version of the manuscript was read and approved by all the authors.

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Regressive Autism Spectrum Disorder: High Levels of Total Secreted Amyloid Precursor Protein and Secreted Amyloid Precursor Protein-α in Plasma

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Autism spectrum disorder (ASD) is a complex neurodevelopmental disorder characterized by social communication difficulties, repetitive behaviors, and parochial interests. Individuals with regressive ASD (RA), a unique subtype, have poor outcomes. Moreover, there are currently no validated blood-based biomarkers for ASD, hindering early diagnosis and treatment. This study was the first to examine plasma levels of total secreted amyloid precursor protein (sAPPtotal), secreted amyloid precursor protein-α (sAPPα), and secreted amyloid precursor protein-β (sAPPβ) in children diagnosed with RA ($n = 23$) and compare them with the levels in age-matched children with non-regressive ASD (NRA) ($n = 23$) and typically developing (TD) controls ($n = 23$). We found that sAPPtotal and sAPPα levels were significantly higher in children with RA than in children with NRA or in TD controls. In contrast, no difference was observed in sAPPβ levels. In conclusion, increased plasma levels of sAPPtotal and sAPPα may be valuable biomarkers for the early identification of ASD regression. Prospective studies will be conducted using a larger sample to further investigate these differences.

Keywords: autism spectrum disorder, regression, amyloid precursor protein, sAPPα, biomarkers

INTRODUCTION

Autism spectrum disorder (ASD) is a neurodevelopmental disorder that emerges in early childhood and is characterized by social communication difficulties, repetitive behaviors, and parochial interests (1). Since the first description by psychiatrist Dr. Sukhareva, ASD has evolved from a rare to a widespread disease, with a prevalence of ∼1.85%, and has become a major public health problem affecting social and economic development (2, 3). Overall, ASD has a serious impact on individuals, families, and societies.

The etiology and phenotypes of ASD are heterogeneous and determined by a complex combination of genetics and the environment (4, 5). At present, the diagnosis of ASD depends on behavioral descriptions and characteristic observations (6), with the average age at diagnosis being 5 years old (7). According to one prospective study, children with ASD exhibit social abnormalities and stereotyped behaviors at 6 months, but these subtle changes are usually ignored by parents (8). Early screening and diagnosis of ASD are very challenging, and the American Academy of Pediatrics recommends that children be screened early and continuously tested before the age of 2 years (9). Although behavioral interventions can improve outcomes, there are no drugs that completely alleviate the symptoms of ASD (10, 11). Importantly, earlier and more frequent behavioral interventions for autism lead to better outcomes (11). Notably, individuals with regressive ASD (RA), a complex subtype of the ASD phenotype, consistently have poor outcomes (12, 13), which may be related to the fact that individuals with RA show poorer language development, more severe autism, and lower intellectual function than those with non-regressive ASD (NRA) (14) as well as to the neurological and pathological bases of regression. At present, RA is a hot research topic.

Although the complex phenotypic causes and pathogenesis of RA have been explored for more than a century, no conclusions have yet been drawn (15). Tan et al. reported that the incidence of RA is up to 30% and that it generally occurs at the age of 19.8 months. Among individuals with RA, 20% exhibit language regression, 40% present language/social regression, 30% show mixed regression, and 27% exhibit unspecified regression (16). A recent prospective report pointed out that information reported by parents is only the tip of the iceberg and that the actual incidence of RA is as high as 80% (8). It has also been reported that immune disorders or neuroinflammation may be involved in the etiology of regression (17, 18). The only evidence involves data from metabonomics and immunology studies of older children (19, 20), and there are few biomarkers in early childhood in RA. Therefore, major goals are identifying early specific biomarkers and diagnostic tools for ASD and its subtypes before core symptoms and regression emerge.

Previous studies have identified 206 autism-susceptibility genes that converge on the amyloid precursor protein (APP) metabolic pathway (21). APP protein is a glycoprotein secreted by glial cells and neurons that promotes neuronal proliferation and migration, cell adhesion, and synapse formation (22, 23). In the non-amyloidogenic pathway, APP is cleaved by α- and γ-secretase liberates secreted APP-α (sAPPα) and p3 peptide (24). However, if APP is initiated by β- and γ-secretase, then secreted APP-β (sAPPβ) and neurotoxic Aβ peptides are generated, which are often involved in Alzheimer's disease (AD) and neurodegeneration (25–27). Notably, if APP is cleaved by γ-secretase at the C-terminus of the Aβ domain, secreted APP-γ (sAPPγ) may be released, and sAPPα, sAPPβ, and sAPPγ comprise total secreted APP (sAPPtotal) in human plasma (28). Plasma levels of sAPPtotal in individuals with severe and aggressive autism, a subtype of autism, are reportedly two or more times higher than those in children without autism (29). Moreover, sAPPα-overexpressing mice exhibit autism-like behavior and reduced social and exploratory behavior (30). Nevertheless, it remains unclear whether APP and its metabolites are involved in the pathophysiological pathways underlying other autism phenotypes.

RA is a form of neurodegeneration that emerges only in childhood (31), and clarifying the specific pathophysiological pathway associated with regression, which may help identify early biomarkers for autism and other neurodegenerative diseases (such as AD), would be a major advance in the field of neurodevelopment and neurodegeneration. Here, we investigated differences in the levels of sAPP isoforms in the plasma of children with and without RA. The identification of early specific biomarkers and associated pathophysiological pathways may guide the identification, diagnosis, and intervention before the emergence of the core symptoms and regression of ASD.

Abbreviations: ASD, autism spectrum disorder; RA, regressive autism; NRA, nonregressive autism; APP, amyloid precursor protein; sAPPα, secreted APP-α; sAPPβ, secreted APP-β; sAPPγ, secreted APP-γ; sAPPtotal, total secreted APP; Aβ, amyloid-β; AD, Alzheimer's disease; ChiCTR, Chinese Clinical Trial Registry; DSM-5, Diagnostic and Statistical Manual of Mental Disorders, 5th edition; CARS, Childhood Autism Rating Scale; CDD, childhood disintegrative disorder; TD, typically developing; LTD, long-term depression; iNOS, inducible nitric oxide synthase; BBB, blood-brain barrier.

MATERIALS AND METHODS

Ethics Statement

The study protocol was approved by the Ethics Committee of the Children's Hospital of Chongqing Medical University (2019; Institutional Review Board Study Approval No. 292) and registered in the Chinese Clinical Trial Registry (ChiCTR) (registration number ChiCTR2000031194). The parents of all subjects provided written informed consent and their agreement for participation in our study. This study conformed to the Declaration of Helsinki.

Participants and Blood Collection

In total, 69 children aged 1.75–5.08 years who were treated at the Pediatrics Department of Children's Hospital of Chongqing Medical University were examined in this case-control study. Children with ASD ($n = 46$) had a confirmed diagnosis of autism according to the clinical criteria in the Diagnostic and Statistical Manual of Mental Disorders, 5th edition (DSM-5), as diagnosed by a proficient clinical psychologist, developmental pediatrician, or child psychiatrist. Using the Childhood Autism Rating Scale (CARS), we further determined symptoms of ASD, and those with CARS scores $\geq$30 were included in the autism group (32). The judgment of ASD's regressive behavior in this study refers to the definition of the same type of research articles (14, 15, 33, 34), and interviews with the parents or caregivers on the child's development process. Notably, some children diagnosed with ASD initially show a period of apparently typical development followed by a considerable loss of previously established skills, a phenomenon termed "regression". Regression is defined as the loss of one or more developmental skills in the areas of personal-social abilities, gross motor performance, and/or fine motor

performance after those skills have been acquired and maintained for 3 months. For example, parents of an 18-month-old boy might be asked if the child used his index finger to indicate his needs. If the parents said the child used to but had stopped doing so, then they were asked when the ability had appeared and whether it had lasted more than 3 months before disappearing. If the parents answered yes, the child would be identified as having undergone regression in personal-social skills. Another type of regression is language regression, defined as the loss of more than five spoken words used communicatively in children over 18 months of age.

Exclusion criteria included participants comorbid with other developmental disorders or psychiatric diseases (e.g., Rett syndrome, cerebral palsy, chronic seizures, and other congenital diseases). Children with autism who had experienced regression and lost acquired skills or knowledge for at least 3 months were included in the RA group ($n = 23$, 3.16 ± 0.77 years old); age- and sex-matched children who did not experience regression were included in the NRA group ($n = 23$, 3.15 ± 0.74 years old). Among those who experienced regression, 12 exhibited language regression, four social regression, four mixed regression, and three another type of regression. Age- and sex-matched typically developing (TD) volunteers composed the control group ($n = 23$, 3.16 ± 0.88 years old) (**Table 1**). Blood samples were collected, separated, and stored in strict accordance with the requirements of the experiment. The collection tubes containing EDTA and blood were centrifuged at 4°C at 1,000 × g for 10 min, after which the top layer of plasma was carefully removed and transferred to an enzyme-free 1.5 ml Eppendorf tube. The plasma was centrifuged at 4°C for 12 min and divided into equal portions to store at −80°C for further analysis. It should be noted that the plasma aliquots underwent no more than 1 freeze cycle.

TABLE 1 | Subject demographics.

	Autism spectrum disorder subtype			Typically developing ($n = 23$)
	Total ($n = 46$)	Regression ($n = 23$)	No regression ($n = 23$)	
Age (y) (mean ± SD)	3.17 ± 0.75	3.16 ± 0.77	3.15 ± 0.74	3.16 ± 0.88
Sex, *n* (%) Male	38 (82.60)	19 (82.60)	19 (82.60)	19 (82.60)

Follow-Up

After all the non-regressive children underwent protein measurements, their developmental progress was investigated by telephone follow-up every 3 months until they reached the age of 36 months. Children who underwent regression during follow-up were defined as having regressive ASD. The follow-up telephone calls asked the following questions:

(1) Did the child have gross motor, fine motor, or personal-social skills that suddenly disappeared or failed to progress after being mastered and maintained for more than 3 months, as opposed to being lost within a short period after the abilities appeared? Examples of personal-social skills were the ability to put on clothes and shoes with the help of their parents and button their clothes. Examples of gross motor skills were the ability to ride tricycles, jump on one foot, stand on one foot 2 out of 3 times for 5 s, etc. Examples of fine motor skills were the ability to build eight-story towers, correctly choose the longer of two line segments three out of three times, copy a drawing of a cross, etc.
(2) Did the child lose more than five spoken words that had once been used communicatively? Examples include the ability to say one's name, understand three to four prepositions, and speak a pair of antonyms.

On the other hand, it is generally believed that regression that occurs after 3 years of age should be defined as childhood disintegrative disorder (CDD) (35, 36). To reduce confounding factors, we generally do not include children with ASD who experience regression after age 3.

Analysis of Plasma sAPPtotal, sAPPα, and sAPPβ Levels

Concentrations of sAPPtotal, sAPPα, and sAPPβ were detected by ELISA, as described by Erickson et al. (37–39). Plasma sAPPtotal concentrations were measured in duplicate using a commercial ELISA kit (IBL, Gunma, Japan). A 50 μl volume of plasma was diluted with 300 μl enzyme immunoassay buffer and mixed evenly. A 100 μl volume of the mixture was added to wells precoated with capture monoclonal anti-human APP (R12A1) and incubated overnight at 4°C after covering it with a plate lid. After washing several times, HRP-conjugated monoclonal anti-human APP (R101A4) was added to all wells and incubated the precoated plate for 30 min at 4°C after covering it with a plate lid. After several washes and the addition of the chromogen substrate, the colorimetric signal was detected at 450 nm using a microplate reader (Thermo). A standard curve was prepared by using known amounts of recombinant human APP protein, and the concentration for unknown samples was read from the standard curve. Levels of the other secreted APP isoforms were similarly measured using ELISA kits (IBL, Gunma, Japan).

Statistical Analysis

All data were analyzed by SPSS 25 and R language statistical analysis software, and the Shapiro–Wilk test was used to test the normality of all data sets. A descriptive analysis is presented as the means (standard deviation) or medians (interquartile ranges). The Friedman test was used to compare levels between groups; the Bonferroni *post-hoc* test was employed for *post-hoc* analyses between groups. Adjusted p-values <0.05 were considered statistically significant. Receiver operating characteristic (ROC) curve analysis was performed to define the discriminatory value of the sAPPtotal and sAPPα proteins to separate RA from NRA

and TD. To verify the independent samples, we present the data in boxplots.

RESULTS

In our study, the sAPPtotal and sAPPα levels were significantly higher in the RA group than in the NRA and TD groups. Levels of sAPPtotal (**Figure 1A**) and sAPPα (**Figure 1B**) were different among diagnoses: TD < NRA < RA. In contrast, no differences in sAPPβ levels were observed in any two of the three groups. Individual results are presented in **Figure 1**. sAPPtotal levels were significantly higher in the RA group than in the NRA group ($p = 0.001$) or the TD group ($p = 0.002$). Interestingly, no significant differences were observed between the NRA and TD groups. Furthermore, RA showed higher sAPPα levels than NRA ($p = 0.024$), with no significant differences between NRA and TD. More remarkably, there were no differences in the sAPPβ levels of any two of the three groups ($p > 0.05$).

To better characterize the balance between the amyloidogenic and non-amyloidogenic pathways in autism, sAPPα/sAPPtotal and sAPPβ/sAPPtotal ratios were measured for each group. However, no significant differences were observed between any two of the three groups ($p > 0.05$) (**Figure 2**).

The use of sAPPtotal and sAPPα proteins as early specific biomarkers has predictive power to identify membership in the RA, NRA, and TD groups. Individual results are presented in **Figure 3**. The area under the ROC curve (AUC) of sAPPtotal for distinguishing RA from TD was 0.779, and the predictive AUC of independent sample validation was 0.75. ROC curve analysis revealed an AUC of 0.687 for the sAPPα protein to separate RA from TD, and the predictive AUC of independent sample validation was 0.71. In the RA and NRA groups, the AUC measurement for the sAPPtotal protein was 0.677 to separate RA from NRA, and the predictive AUC of independent sample validation was 0.7. ROC curve analysis revealed an AUC of 0.698 for the sAPPα protein to separate RA from NRA, and the predictive AUC of independent sample validation was 0.67.

DISCUSSION

Heller first defined regression as "dementia infantilis" in the early 20th century, and after more than a century, its mechanism is still unknown (15, 40). One epidemiological investigation found that the risk of AD in autistic patients is 5 times higher than that in individuals without developmental disabilities (41). Notably, the accumulation of Aβ in neurons is enhanced in the brains of idiopathic and dup15q11.2-q13 autism patients (42). APP metabolic pathways may be similar or different in individuals with RA and those with AD, but the relationship is not clear.

In contrast to AD, autism with regression of language, social or other abilities usually occurs in early childhood and gradually improves with age. Given the existence of neurodegenerative symptoms in early childhood in those with RA, we first examined differences between the levels of APP and its metabolites in children with and without RA. The results showed that plasma levels of sAPPtotal and sAPPα were higher in the RA group than in the NRA and TD groups, but there were no significant differences in sAPPβ levels between any two of the three groups.

High levels of sAPPα have been proven to be beneficial in the young adult brain, aged brain, and neurodegenerating brain (43). Indeed, studies on cultured neurons and animals have revealed that in the young brain, a high level of sAPPα aids in neurite outgrowth (44), increases synaptic density (45), stimulates neural stem cell proliferation (46) and differentiation (47), and enhances spatial memory and memory consolidation (48). Furthermore, during aging and neurodegeneration, a high level of sAPPα reduces oxidative stress and cell death (49), rescues synaptic plasticity and synapse number (50) and spatial memory (51), and reduces Aβ plaque deposition (52).

Nevertheless, excessive levels of sAPPα are harmful during the critical time window of brain development. During synaptic formation, excessive sAPPα reduces developmental spine pruning and impairs synaptic long-term depression (LTD), and changes in synaptic development and synaptic plasticity may result in ASD and memory impairment (53–55). In addition, excess sAPPα promotes the expression of inducible nitric oxide synthase (iNOS) in microglia, releasing glutamate and D-serine to cause neurotoxicity (56, 57). Moreover, an increased level of sAPPα during the critical window of development induces overdifferentiation of neural stem cells into astrocytes and enhances the brain immune response by disrupting the IL-6/gp130 pathway, leading to aberrant synaptic connections and brain damage (30).

However, it is worth noting where plasma sAPP, sAPPα, and sAPPβ come from. Based on the present study, we cannot determine whether they come from the brain or the gut. To date, no studies have confirmed whether the sAPPα protein originates from the gut or the brain. On the one hand, we consider that the sAPPα protein may come from the gut. In the last few years, the importance of gut microbiota impairment in the etiopathogenesis of pathologies such as autism and dementia has been raised (58). There has been an emerging interest in the possible role of the gut microbiota as a cofactor in the development of ASD; for example, serotonin is one of the possible links in the gut-brain–microbiome axis in ASD (59, 60). Some studies found that microbiome differences in ASD patients may be related to dietary preferences related to stereotyped behavior or narrow interest, thus weakening the association between ASD and intestinal microbiota (61). However, accumulating evidence has shown a link between alterations in the composition of the gut microbiota and both gastrointestinal and neurobehavioral symptoms in children with ASD (62). Furthermore, studies have shown that gut microbial dysbiosis may lead to the secretion of amyloid, which disturbs gastrointestinal permeability and the blood-brain barrier (BBB). In this way, it may induce neuronal injury and ultimately lead to neuronal death (63–65). However, there is not enough evidence to prove whether the sAPPα protein, as an amyloid precursor protein cleavage product, can cross the BBB and have harmful effects during critical periods of brain development, which will be a very interesting and valuable research direction. On the other hand, we consider that sAPPα proteins may leak from the BBB. Studies have shown that excess sAPPα can activate microglia and astrocytes (30, 57),

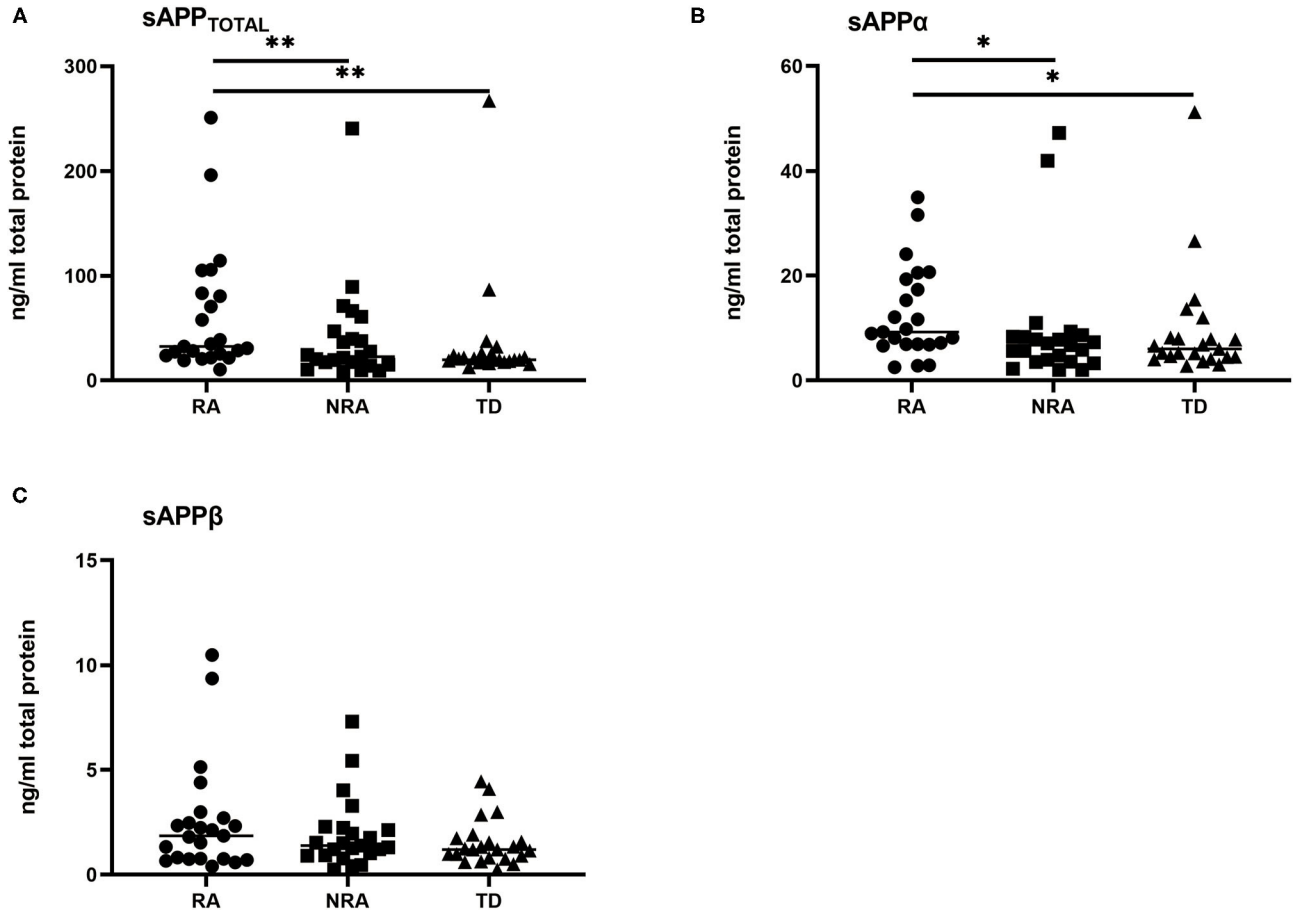

FIGURE 1 | Levels of various secreted APP isoforms in different autism phenotypes and controls. **(A)** RA showed significantly elevated sAPPtotal levels compared with NRA (**$p = 0.001$) and TD (**$p = 0.002$). **(B)** RA had the highest sAPPα levels compared with NRA (*$p = 0.024$) and TD (*$p = 0.024$). **(C)** No significant difference was found in the sAPPβ levels of any two of the three groups. The Friedman test was used to calculate p-values, with Bonferroni *post-hoc* test correction.

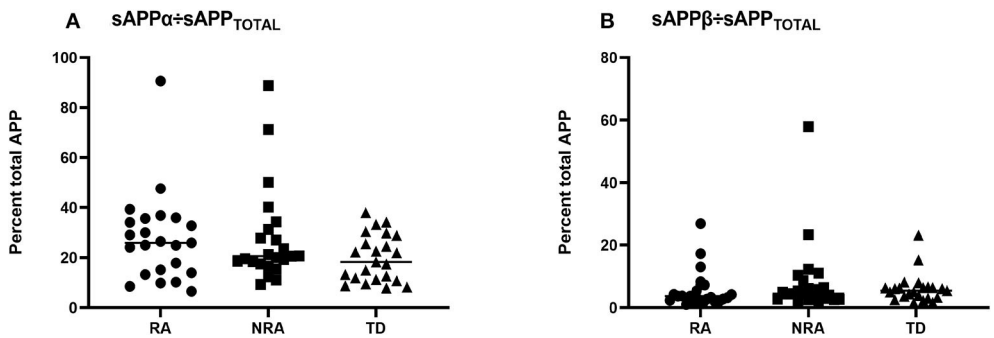

FIGURE 2 | Ratios of the levels of different secreted APP isoforms in children with ASD and controls. To further explore differences in sAPPα, sAPPβ, and sAPPtotal levels, the **(A)** sAPPα/sAPPtotal ratio and **(B)** sAPPβ/sAPPtotal ratio were measured for each group, and there was no difference in any pairwise comparison of the three groups ($p > 0.05$). The Friedman test was used to calculate p-values, with Bonferroni *post-hoc* test correction.

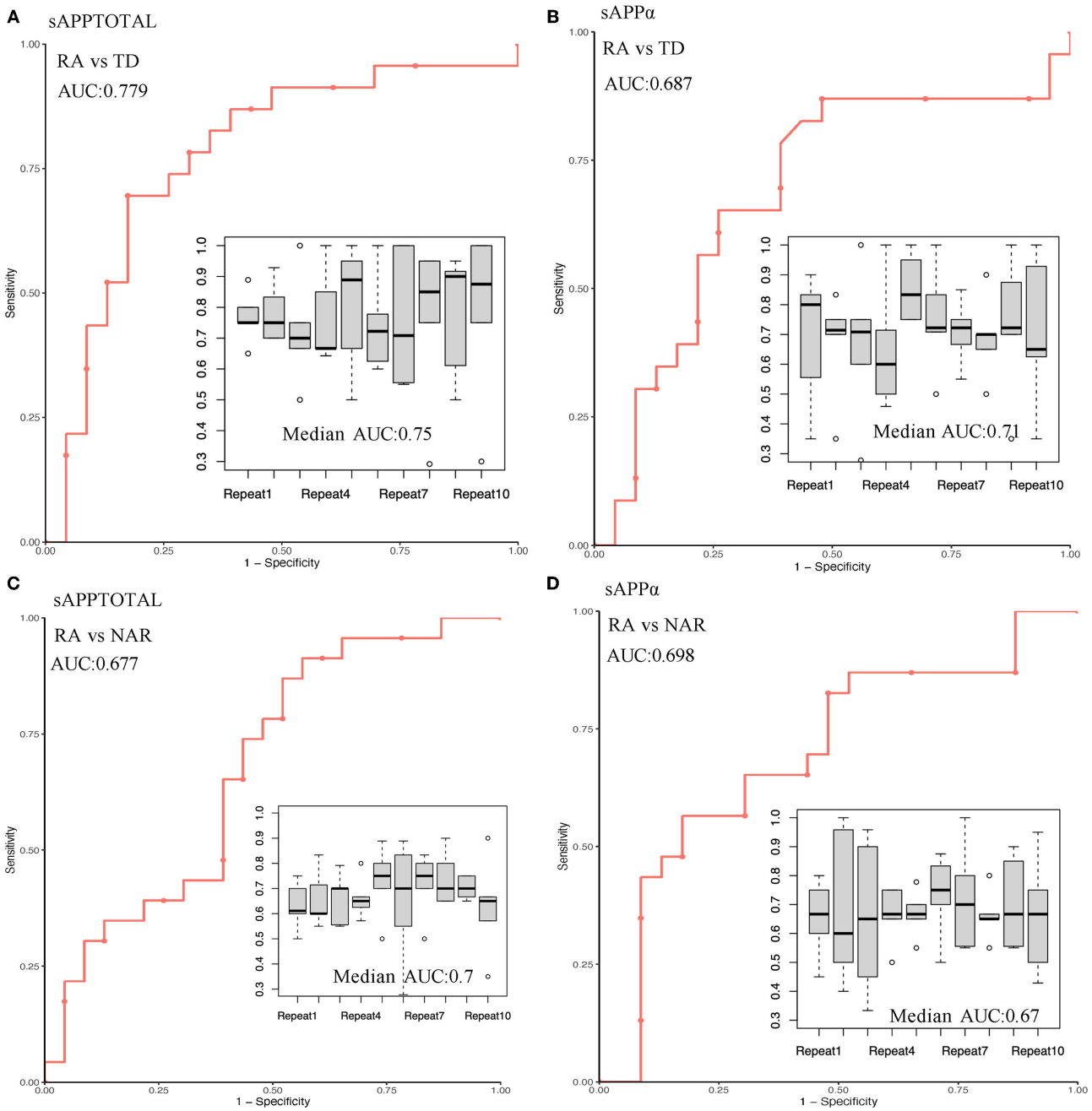

FIGURE 3 | Prediction performance and independent validation of the sAPPtotal and sAPPα proteins in different groups. In the RA and TD groups, AUC measurements for the sAPPtotal and sAPPα protein to separate RA from TD were 0.779 **(A)** and 0.687 **(B)**, respectively. In the RA and NRA groups, AUC measurements for the sAPPtotal and sAPPα protein to separate RA from NRA were 0.677 **(C)** and 0.689 **(D)**, respectively. Repeated independent samples were used for ROC curve verification, and the data are represented by box graphs.

which contribute to BBB dysfunction (66), but it is unclear whether the sAPPα protein will leak from the damaged BBB, which needs to be further verified in animal experiments and will be a very valuable direction for our subsequent research. In conclusion, with the limitations of the current sample, we were able to obtain only peripheral blood in the hope of finding early predictive markers; in our next step, we will verify these markers in animal experiments to explore the source of all sAPP proteins. Significantly, in our study, compared with the RA and TD groups, children with regressive autism had higher sAPPtotal and sAPPα levels. It may be valuable for the early identification of ASD regression.

RA, a distinct subtype of autism in which the non-amyloidogenic pathway may be preferentially active, may be the result of associated pathophysiological changes. In conclusion, we believe that an increase in sAPPα levels may reflect an early increase in the body's responsiveness but that an excessive increase in childhood may lead to neurotoxicity, aberrant synaptic connections, and brain damage. Therefore, inhibiting an abnormal increase in sAPPα may help prevent the regression process. Overall, sAPPtotal and sAPPα levels may serve as biomarkers for the early diagnosis and prognosis of RA.

Ray found that compared to other individuals (67), sAPPβ levels are decreased in individuals with severe autism; however, our study did not detect decreased levels of sAPPβ in children with RA. Notably, the average age of individuals with severe autism in Ray's study was 8.17 years, whereas the average age of children with RA in our study was 3.13 years. A potential explanation for the discrepancy is that the expression of APP and its metabolites differs during the various stages of childhood. It is not clear whether the activity of the toxic metabolic pathway that produces sAPPβ gradually declines with age, the level of sAPPα increases with age, and its physiological effect is beneficial. This hypothesis needs to be verified by prospective cohort studies with longer-term observations in the future.

LIMITATIONS

There are limitations to our study. For example, children were included based on retrospective reports of parents, which may have affected our results. Therefore, a prospective study of children with RA might more accurately reveal the dynamic changes in the levels of APP and metabolites in these individuals.

CONCLUSIONS

Clarifying the common neurobiological mechanisms of neurodevelopmental disorders and neurodegenerative disease may be useful in identifying early disease biomarkers. Importantly, we observed that plasma levels of sAPPα and sAPPtotal were elevated in early childhood in individuals with RA. Increased plasma levels of sAPPtotal and sAPPα may be secondary changes in response to another pathological change that might be the causal pathology. Our study may be valuable biomarkers for the early identification of ASD regression.

Prospective studies will be conducted using a larger sample to further investigate these differences.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of the Children's Hospital of Chongqing Medical University (approval number (2019) IRB (Study) No. 292). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

TL and LC conceived and designed the experiments. XLi, PZ, and QL performed the experiments. BP, YC, YD, HW, XLiu, YY, QF, YZ, ZJ, TY, JC, and QC contributed reagents, materials, analysis tools. XLi and LC wrote the paper. All authors contributed to the article and approved the submitted version.

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Motor Cortex Excitation/Inhibition Imbalance in Young Adults With Autism Spectrum Disorder: A MRS-TMS Approach

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Excitatory/inhibitory imbalance has been suggested as a neurobiological substrate of the cognitive symptomatology in Autism Spectrum Disorder (ASD). Studies using magnetic resonance spectroscopy (MRS) attempted to characterize GABA and Glutamate brain levels in ASD. However mixed findings have been reported. Here, we characterize both neurochemical and physiological aspects of GABA system in ASD by implementing a more comprehensive approach combining MRS and transcranial magnetic stimulation (TMS). A group of 16 young ASD adults and a group of 17 controls participated in this study. We employed one MRS session to assess motor cortex GABA+ and Glutamate+Glutamine (Glx) levels using MEGAPRESS and PRESS sequences, respectively. Additionally, a TMS experiment was implemented including paired-pulse (SICI, ICF and LICI), input-output curve and cortical silent period to probe cortical excitability. Our results showed a significantly increased Glx, with unchanged GABA+ levels in the ASD group compared with controls. Single TMS measures did not differ between groups, although exploratory within-group analysis showed impaired inhibition in SICI5ms, in ASD. Importantly, we observed a correlation between GABA levels and measures of the input-output TMS recruitment curve (slope and MEP amplitude) in the control group but not in ASD, as further demonstrated by direct between group comparisons. In this exploratory study, we found evidence of increased Glx levels which may contribute to ASD excitatory/inhibitory imbalance while highlighting the relevance of conducting further larger-scale studies to investigate the GABA system from complementary perspectives, using both MRS and TMS techniques.

Keywords: magnetic resonance spectroscopy, transcranial magnetic stimulation, autism (ASD), GABA, glutamate

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INTRODUCTION

Inhibitory gamma-aminobutyric acid (GABA) system dysfunction has been hypothesized to contribute to the pathophysiology of a cluster of neurodevelopmental and psychiatric disorders (1, 2). Autism spectrum disorder (ASD) is one of the conditions for which cortical excitatory-inhibitory (E-I) imbalance has been proposed as underlying etiology (3, 4), along with other pathologies with overlapping symptomatology (2, 5, 6).

Evidence from post-mortem and animal studies have strengthen this hypothesis by demonstrating altered markers of glutamatergic and GABAergic neurotransmission (7–9). Most recently, this hypothesis has been addressed in clinical studies using [1H] magnetic resonance spectroscopy (MRS), a non-invasive technique which allows *in vivo* quantification of metabolites in the brain (3, 10). Despite the steady increase of MRS studies trying to indirectly characterize GABAergic system in ASD through the quantification of MRS-derived GABA levels, the demonstration of a consistent pattern has been proved challenging. ASD is a highly heterogeneous neurodevelopmental disorder characterized by marked impairments in social interaction and communication in the presence of repetitive stereotyped behavior, sensory anomalies, and variable levels of intellectual disability (11, 12). This phenotypical variability, probably reflecting distinct neurobiological correlates, along with different methodological approaches across studies has contributed to mixed findings.

Concerning GABA system, studies in pediatric populations have shown reduced levels across several brain regions, including frontal and auditory cortices, motor and sensorimotor areas, anterior cingulate cortex, and cerebellum (13–18), while others reported comparable levels in occipital, anterior cingulate and medial prefrontal cortices (15, 19–22). In adulthood, mixed findings have been reported with some studies showing unchanged GABA levels in different brain regions: visual, auditory, and motor cortices, dorsal and medial prefrontal cortices, superior temporal sulcus and sensorimotor areas (23–28), while others revealed higher levels in the dorsal lateral prefrontal cortex (29) and lower GABA levels in the sensorimotor cortex (30) and supplementary motor area (28). Likewise, findings regarding Glx, which stands for glutamate (Glu) + glutamine (Gln), have been inconsistent, with studies revealing higher (31–33), unchanged (20, 25, 27) or lower (24, 34) levels in both children and adults with ASD when compared with typically developing controls. The link between neurochemical alterations in ASD and cognitive symptomatology has also been explored and GABA levels were associated with sensory impairments (15, 28) and behavioral measures from ASD diagnostic tools (19, 20).

Despite the substantial value that MRS studies brought to the investigation of E-I imbalance in neurodevelopmental disorders, the static measures of GABA and Glutamate levels obtained from this technique are not able to capture the dynamic process involved in cortical excitability (35). Importantly, MRS-derived GABA and Glx levels do not equate directly to GABAergic and Glutamatergic neurotransmission, respectively. Hence, the study of E-I imbalance in ASD could benefit from the inclusion of transcranial magnetic stimulation (TMS) which directly addresses inhibitory and excitatory modulation.

TMS is a non-invasive technique which allows the assessment of cortical excitability through focal brain stimulation (36). Depending on the TMS paradigm, one can tap into different neural circuits associated with both GABAergic and Glutamatergic signaling. Paired-pulse TMS (pp-TMS) is a well-established paradigm to investigate excitatory and inhibitory mechanisms, depending on the interval between the two magnetic pulses administered. Short-interval intracortical inhibition (SICI) is assumed to reflect $GABA_A$ receptor-mediated neurotransmission whereas intracortical facilitation (ICF) is thought to be mediated by glutamatergic N-methyl-D-aspartate receptors and long-interval intracortical inhibition (LICI) seems to reflect inhibition mediated by $GABA_B$ (37, 38). To date, few studies investigated GABAergic neurotransmission in ASD using TMS. A recent systematic review (35) reported five studies measuring motor-evoked potentials (MEP), SICI and LICI in ASD and suggested that SICI is likely to be reduced in ASD, whereas MEP and LICI were comparable between groups.

Studies exploring the relationship between TMS physiological measures of cortical excitability and metabolites levels obtained from spectroscopy have raised the question that each technique measures specific aspects of GABAergic neurotransmission (39, 40). MRS GABA levels are thought to reflect tonic instead of phasic inhibitory processes and may not be associated with synaptic activity (41), in opposition to TMS (42). Additionally, the literature also shows that interventional TMS protocols can induce GABA changes in the expected directions (43–46) suggesting that there is a link between brain metabolism and inhibition and facilitation (41). This points to the putative complementary nature of both techniques and highlights its relevance for the study of disease mechanisms. Although some studies explored the link between brain metabolites levels and measures of cortical excitability using combined MRS-TMS in healthy subjects (39, 40), little is known in disease context.

In the current study, we aimed to comprehensively investigate the E-I balance in ASD by testing the mechanistic role of GABA neurotransmission from the neurochemical and physiological points of view. We hypothesize that GABA and Glx neurotransmitters levels as well as excitability patterns are altered in ASD, in line with the prediction of expected changes in E-I. To our knowledge, this is the first study combining both MRS and TMS techniques, in the same clinical and control groups, to address the E-I imbalance hypothesis in ASD young adults. With this in mind, we believe that our findings might also inform about the complementary nature of MRS-TMS by elucidating their relationship in both health and disease.

MATERIALS AND METHODS

Participants

Thirty-four participants were recruited, including a group of 17 young male adults with ASD and a control group with 17 typically developing (TD) male participants. One of the ASD participants was not able to collaborate in both MRI and TMS data acquisitions and was excluded from the study. As a result, 16 ASD participants and 17 TD participants were included in the analyses.

ASD participants were recruited from a database used in previous studies (47, 48) and in collaboration with local ASD associations. All ASD participants obtained positive results on the gold standard diagnostic instruments, namely parental or caregiver interview [Autism Diagnostic Interview-Revised, ADI-R (49)] and direct structured proband assessment [Autism Diagnostic Observation Schedule, ADOS (50)], and met the

TABLE 1 | Demographic and diagnostic data.

	ASD ($n = 16$)		CTRL ($n = 17$)	
	Mean (S.E.)	**Range**	**Mean (S.E.)**	**Range**
CA (years)	20.6 (0.9)	17–30	22.9 (0.8)	16–29
Education (years)*	12.4 (0.4)	11–17	15.0 (0.5)	10–17
Sex (male:female)	16:0		17:0	
Handedness (right:left)	13:3		15:2	
Full-scale IQ*	105.4 (2.7)	86–119	120.1 (2.5)#	108–129
VIQ*	104.6 (3.6)	70–126	120.9 (2.6)#	105–137
PIQ	106.8 (3.0)	91–129	114.9 (3.6)#	101–137
ADI-R				
Reciprocal social interactions	15.8 (1.0)	8–25		
Language/ Communication	10.4 (0.9)	3–18		
Repetitive behaviors/Interests	5.4 (0.5)	3–9		
Developmental delay	2.1 (0.5)	0–5		
ADOS				
Total result	9.3 (0.3)	7–12		
Communication	2.8 (0.2)	2–5		
Social interaction	6.4 (0.3)	5–8		

*ASD, Autism Spectrum Disorder group; CTRL, Control group; S.E., Standard Error; CA, Chronological Age; IQ, Intelligence Quotient; VIQ, Verbal Intelligence Quotient; PIQ, Performance Intelligence Quotient; *$p < 0.05$; #$n = 11$.*

current diagnostic criteria for ASD as assessed by the Diagnostic and Statistical Manual of Mental Disorders [5th ed.; DSM-5 (12)]. Exclusion criteria included genetic syndrome, neurological or psychiatric comorbidities, history of traumatic brain injury, epilepsy, contraindications to MR scanning or TMS and severe learning disabilities (full-scale intellectual quotient <85). None of the participants were diagnosed with ADHD, OCD, anxiety or mood disorders. Three ASD participants were under chronic medication for ASD-related symptomatology (methylphenidate $n = 2$; risperidone $n = 1$) and were instructed to maintain the treatment as usual.

TD participants were recruited from the local community, had no history of psychiatric and/or neurological illnesses, or contraindication to MRI or TMS acquisitions and were not taking any medication.

Participants included in the study received the Portuguese version of the Wechsler Adult Intelligence Scale, WAIS-III (51) to perform intellectual quotient (IQ) assessment. Handedness was evaluated with the Edinburgh Inventory (52). Demographic and diagnostic measures are detailed in **Table 1**.

ASD and control groups were matched for chronological age ($t_{(31)} = -1.914$, $p = 0.065$), handedness ($p = 0.656$), and performance IQ ($t_{(25)} = -1.752$, $p = 0.094$). Total IQ ($t_{(25)} = -4.035$, $p = 0.000$), verbal IQ ($t_{(25)} = -3.699$, $p = 0.001$) and level of education (U = 48.000, $p = 0.001$) revealed expected differences between groups given the intellectual profile described in ASD (53, 54).

The study procedures were revised and approved by the Ethics Committee of the Faculty of Medicine of the University of Coimbra and are in accordance with the Declaration of Helsinki. All participants gave verbal informed consent. Moreover, we obtained written informed consent from participants, or their parents when appropriate.

Procedures

The study encompassed a 1-day visit in which demographic and intellectual assessment was performed followed by MRS and TMS data acquisition. Although we did not expect TMS after-effects, stimulation was always performed after MRS to avoid possible interferences on neurochemical data.

Proton Magnetic Resonance Spectroscopy (1H-MRS)

We acquired Magnetic Resonance Imaging (MRI) data on a Siemens MAGNETOM Trio Tim 3T (Erlangen, Germany), equipped with a 12-channel birdcage head coil, at our facilities (ICNAS/CIBIT, University of Coimbra). During the whole MRI experiment, movement was controlled by the continuous monitoring of the eyes positioning [Eyetracker: SensoMotoric Instruments (SMI), Teltow, Germany].

Structural images were obtained through a high-resolution T1-weighted 3D MPRAGE (Magnetization Prepared Rapid Acquisition Gradient Echo) sequence, with a voxel size of $1 \times 1 \times 1$ mm^3 (FOV, field of view = 256×256 mm^2; 176 slices; TR, repetition time = 2,530 ms; TE, echo time = 3.42 ms; TI, inversion time = 1,100 ms; FA, flip angle = 7°).

To estimate the levels of GABA and Glx metabolites in the dominant motor cortex with both MEGA-PRESS and PRESS sequences, respectively, we first ran a functional localizer to select the motor region activated by a finger-tapping task (as in Silva et al. (55), including both synchronous and asynchronous tapping, at particular frequencies), and subsequently placed a $3 \times 3 \times 3$ cm^3 voxel on the corresponding location for both MEGA-PRESS and PRESS sequences. We marked the region-of-interest including the activation map and used the center coordinates of the activation to position the voxel. Small adjustments were performed to avoid including skull or the ventricles in the voxel, which could affect our data. An example of voxel positioning is presented in **Figure 1**.

Quality of the data was assessed following the recommended guidelines consensus (56), as detailed below.

GABA Quantification

The MEGA-PRESS (MEshcher-GArwood Point RESolved Spectroscopy) (57) sequence was employed (TR = 1,500 ms; TE = 68 ms; 196 averages and 1,024 points) to estimate GABA levels through a J-difference editing technique. Unsuppressed water spectra (16 averages) were also obtained in the same voxel to determine water-scaled levels. Metabolite quantification was carried out in Gannet (v. 3.1.5), a MATLAB-based GABA Analysis Toolkit (58). To improve signal-to-noise ratio (SNR), we used exponential line broadening at 3 Hz. We then processed the time-resolved data into the frequency domain with Fast Fourier Transform. We performed RobustSpecReg alignment to correct both frequency and phase to improve the quality

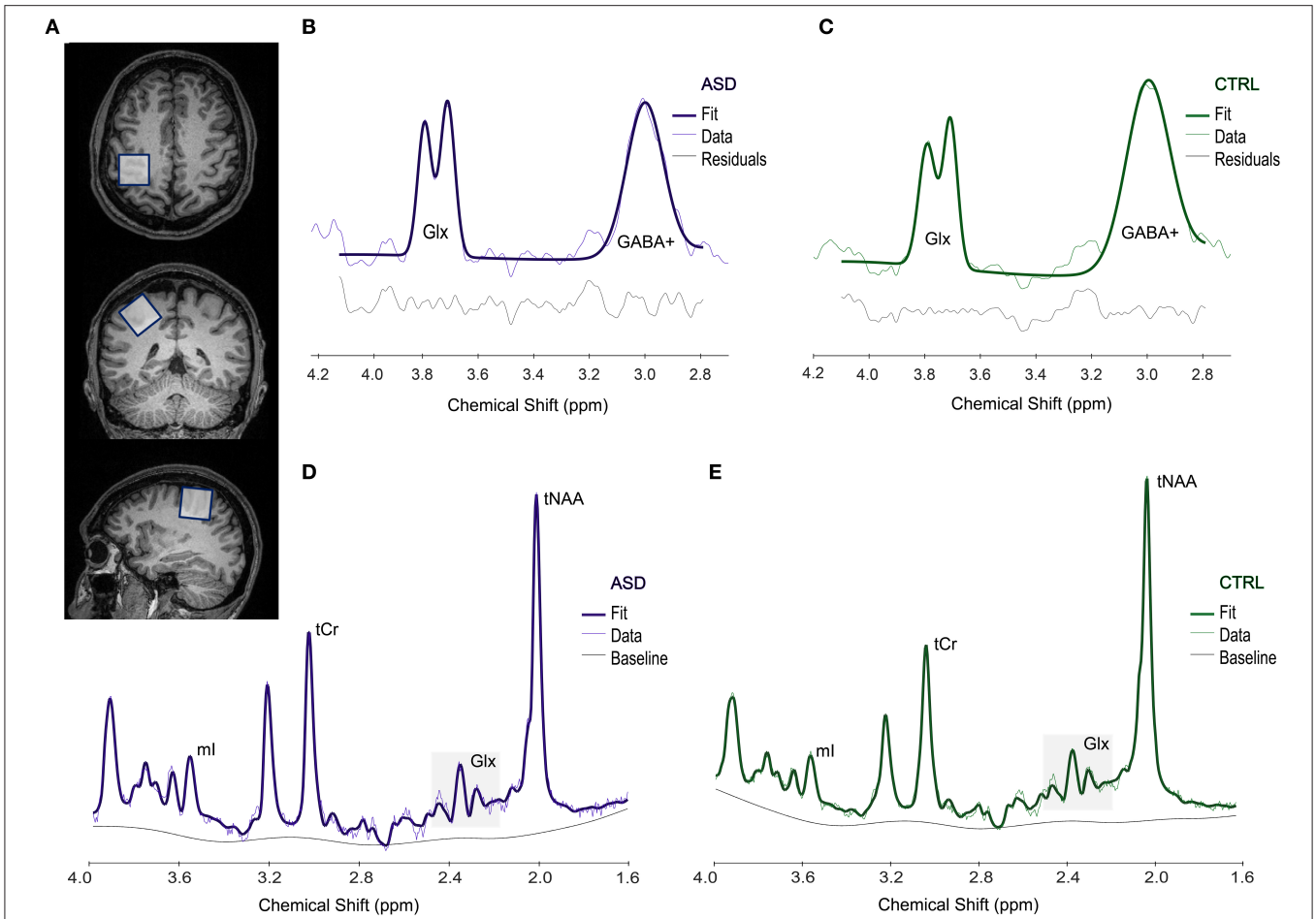

FIGURE 1 | Example of the voxel placement in the motor area **(A)**. Upper panel shows a representative spectrum from an ASD **(B)** and a CTRL **(C)** participant, obtained from Gannet and used to estimate GABA+ levels. Lower panel shows a representative spectrum processed in LCModel, used to measure Glx levels in both ASD **(D)** and CTRL **(E)** groups. ASD, Autism Spectrum Disorder group; CTRL, control group; Glx, glutamate+glutamine; GABA+, gamma-aminobutyric acid; mI, myo-inositol; tCr, total creatine; tNAA, total N-acetylaspartate; ppm, parts per million.

of the spectra and applied eddy current correction to water and metabolite signals. In addition, we filtered the data with a Hankel singular value decomposition (HSVD) filter to reject residual water signal from the difference spectra. A non-linear least-squares fitting approach was used for the integration of the edited GABA (~at 3.00 ppm) through a Gaussian model applied to the difference spectrum.

We used GABA+/water levels (i.u., institutional units) and corrected the metabolite levels for voxel composition by the alpha tissue correction method (59). For this purpose, we estimated the fractions of gray matter (GM), white matter (WM) and cerebrospinal fluid (CSF) in the voxel through coregistration and segmentation by Gannet and SPM12 toolbox (Wellcome Trust Centre for Neuroimaging, Institute of Neurology, UCL, London, UK). To acknowledge a possible contribution from macromolecules and homocarnosine to GABA estimation (60), we reported our levels as GABA+. Therefore, in this work, we report corrected GABA+/water levels.

Full width at half maximum (FWHM) and SNR for the entire spectrum were determined and the overall quality of the data was evaluated by visual inspection, conducted by two researchers. Data with unsatisfactory quality or with a GABA+ fit error superior to 10% were not included for further analyses. An example of representative spectra is presented in **Figure 1**.

Glx Quantification

We applied the PRESS (Point RESolved Spectroscopy) sequence (TR = 2,000 ms; TE = 35 ms; 46 averages and 1,024 points; unsuppressed water: 16 averages) in the same location, and used LCModel v. 6.3-1M (61) to estimate Glx levels. Water scaling and eddy-current correction were performed, and spectra were obtained from 1.6 to 4.0 ppm to reduce artifacts due to the contamination from lipids and macromolecules. In this work, we present the corrected values for Glx/water, considering voxel tissue composition, namely GM, WM and CSF fractions, as fully detailed in Naaijen et al. (62). We assessed FWHM and SNR for the entire spectrum and excluded from the analyses those

spectra with bad quality identified by visual inspection or with Glx Cramér-Rao Lower Bounds (CRLB) > 10%. For an example of selected spectra, please see **Figure 1**.

Transcranial Magnetic Stimulation

Transcranial magnetic stimulation was performed using a MagPro X100 magnetic stimulator (MagVenture, Denmark), equipped with a MCF-B65 figure-of-eight coil (MagVenture, Denmark). All participants wore earplugs and were resting in a comfortable armchair. The hotspot was defined as the region in the dominant primary motor cortex (M1) with the greatest response to the stimulation pulses. The coil was placed over the hotspot, tangentially to the scalp, at 45° to the sagittal plane. The electromyographic (EMG) signal was recorded in the first dorsal interosseous (FDI) muscle by Ag/AgCl electrodes (Biopac Systems, CA, USA), in a belly-tendon montage, coupled to an EMG 100C amplifier and connected to a BIOPAC MP-150 system (Biopac Systems, CA, USA), with a gain of 1,000, to register motor-evoked potentials. The Acqknowledge 4.2 software (Biopac Systems, CA, USA) was used to acquire EMG signal at a 2.5 kHz sampling rate and to process data.

Paired-Pulse (pp-TMS)

We first determined SI1mV, by testing different intensities until we found the minimum individual intensity that elicited motor-evoked potentials with a peak-to-peak amplitude of at least 1mV in 5 or more trials out of a total of 10 consecutive trials. For both SICI and ICF we applied a suprathreshold test pulse at 120% SI1mV, preceded by a subthreshold conditioning pulse (80% of SI1mV). In contrast, for LICI both the conditioning and test pulses were applied with an intensity of 100% SImV. We studied different protocol-dependent inter-stimulus intervals (ISIs): 1, 3 and 5 ms to study SICI; 10, 15 and 20 ms for ICF; and 50, 100 and 150 ms to assess LICI. For each protocol, we delivered 10 pairs of pulses for each ISI, in a random order, and 10 single-pulses (baseline) at the same intensity of the test stimulus, as described by De Beaumont et al. (63). We identified motor-evoked potentials and determined their peak-to-peak amplitude using an in-house script. LICI measures were excluded from the analyses because the substantial number of missing values hindered an adequate statistical comparison between groups. All measurements were validated by visual inspection, trial-by-trial. Furthermore, for each individual, we normalized the amplitudes obtained for each ISI, by calculating paired-pulse:baseline MEP amplitude ratios.

Input-Output or Recruitment Curve (I-O Curve)

Resting motor threshold (rMT) was defined as the lowest intensity that elicited at least 5 MEPs with peak-to-peak amplitude $\geq$ 50 μV out of 10 consecutive single-pulses. We constructed an input-output curve for each participant, using the following intensities: 90, 100, 110, 120, 130, and 140% of the individual rMT, as reported by De Beaumont et al. (63). Sixty pulses (10 per intensity) were applied in a randomized order. We determined the maximal peak-to-peak amplitude of MEPs, the stimulation intensity required to elicit a half-maximal MEP (S50) and the curve slope.

Cortical Silent Period (CSP)

The resting motor threshold was also used to select the intensity for the cortical silent period protocol. We delivered to the dominant M1 a single-pulse at 130% of rMT, in the middle of a 10-s voluntary contraction of the contralateral hand, at 20% of the participant's maximal force. The force was controlled online by the participant and an investigator, through the inspection of a hand-held digital dynamometer. This procedure was repeated for 10 trials, with an inter-trial interval of 10 s included to avoid fatigue and its potential effects in intracortical $GABA_B$-mediated inhibition (64). We studied relative and absolute silent period durations, measured by two authors, with the onsets and offsets being defined according to the work from Säisänen et al. (65). When present, the breakthrough EMG activity was counted as part of the CSP and included in the measurement (66). Additionally, CSP:MEP ratios were calculated to reduce the interindividual variability intrinsic to CSP durations (66).

Statistical Analyses

We conducted statistical treatment of all data in the SPSS Statistics software (version 27; IBM SPSS Statistics, IBM Corporation, Chicago, IL), and established a significance level of 0.05. Data normality was evaluated with the Shapiro-Wilk test and extreme outlier values excluded. We ran independent samples *t*-test for between-groups comparisons or its non-parametric equivalent Mann-Whitney U, when appropriate, reporting the exact *p*-values. Welch's *t*-test was reported wherein sample size was distinct between groups (IQ measures). Handedness was compared between groups with the Fisher's exact test. Within-group exploratory analysis performed for the paired-pulse TMS paradigm was carried out by applying the Wilcoxon test, since data were not normally distributed. Benjamini-Hochberg false discovery rate (FDR) method (67) was used to correct for multiple comparisons ($\alpha = 0.05$).

In an exploratory approach, we investigated the correlations between ADI-R (Reciprocal Social Interaction, Language Communication and Repetitive Behaviors indices)/ADOS (Communication and Interaction indices) and MRS measures (GABA+ and Glx) for the ASD group, with Spearman's rho. The same test was applied to investigate possible correlations between TMS (input-output curve: slope, S50 and max MEP; ppTMS: SICI and ICF measures; CSP: absolute and relative) and MRS (GABA+ and Glx) measures, for both the ASD and CTRL groups. In those cases, wherein we found significant correlations only for one group (CTRL), we provide a comparison of effects, by testing the slope of the regression line and assuming that in the group that did not reach significance for the studied correlations (ASD) the slope is null. Given the exploratory nature of the correlation analyses, we did not correct for multiple comparisons.

RESULTS

Proton Magnetic Resonance Spectroscopy (1H-MRS)

Concerning metabolite levels, tissue-corrected Glx levels in the motor cortex were significantly increased in ASD when

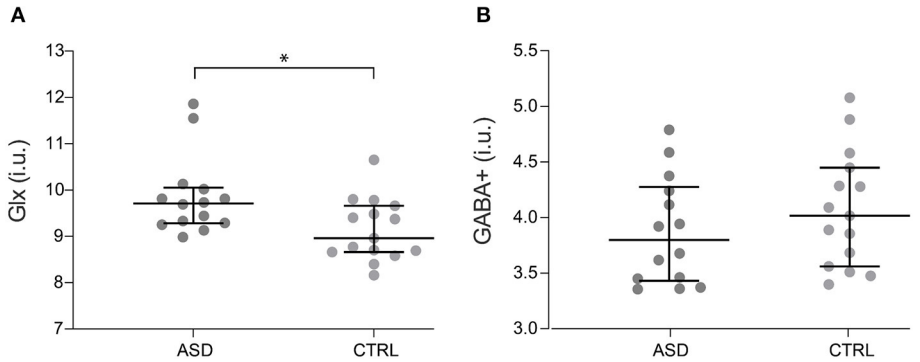

FIGURE 2 | Glx **(A)** and GABA+ **(B)** levels for ASD ($n = 14$) and control participants ($n = 15$). Dots represent individual values and horizontal lines depict median and 95% confidence interval. *$p < 0.05$. ASD, Autism Spectrum Disorder group; CTRL, control group; GABA+, gamma-aminobutyric acid; Glx, glutamate+glutamine.

compared to control participants, with differences surviving FDR correction ($Z = -2.400$, $p = 0.016$, **Figure 2A**), while corrected GABA+ levels ($t_{(27)} = -1.032$, $p = 0.311$, **Figure 2B**) did not show significant differences between groups.

In addition, Glx levels in patients correlated with both the Social Interaction score from ADOS (rs $= -0.569$, $p = 0.034$) and ADI-R Repetitive Behaviors/Interests component (rs $= 0.601$, $p = 0.023$). We did not find significant correlations between IQ scores and any of the MRS measures explored in the study ($p > 0.05$), which ruled out an effect of IQ in our results.

The quality of the spectra included in the analyses was partially ensured by maintaining GABA+ Fit Error and Glx Cramér-Rao values below 10% for MEGA-PRESS and PRESS sequences, respectively (**Table 2**). Additionally, FWHM linewidth (MEGA-PRESS: $t_{(27)} = -0.032$, $p = 0.974$ and PRESS: Z $= -0.628$, $p = 0.543$) and SNR (MEGA-PRESS: $t_{(27)} = -0.272$, $p = 0.788$ and PRESS: $t_{(28)} = 0.383$, $p = 0.705$) indices were equivalent between groups.

Voxel tissue composition was similar between ASD and control groups (GM: $t_{(27)} = 0.797$, $p = 0.432$; WM: $t_{(27)} = -0.952$, $p = 0.350$; CSF: $t_{(27)} = 0.471$, $p = 0.642$), excluding the impact of tissue composition in the metabolite levels explored in this study (**Table 3**).

Transcranial Magnetic Stimulation

Concerning the paired-pulse protocol, within-subject analysis, where the mean MEP peak-to-peak amplitude of the conditioned stimulus was compared to the mean peak-to-peak amplitude of baseline pulses, revealed that all interstimulus intervals (ISIs) of SICI and ICF induced the expected significant inhibition and facilitation, respectively, in the control group ($p < 0.05$, with the results surviving FDR correction). Interestingly, the same effect was not verified for the ASD group at 5 ms ISI ($Z = -1.156$, $p = 0.278$) (**Figure 3**). Between-group differences were not observed ($p > 0.05$). The other single TMS measures were not informative.

Multimodal Correlations

Since TMS and MRS are both used in the study of cortical excitability, we investigated for a multimodal correlation between these measures.

TABLE 2 | MRS data quality parameters.

	ASD ($n = 14$)		CTRL ($n = 15$)	
	Mean (S.E.)	**Range**	**Mean (S.E.)**	**Range**
MEGA-PRESS				
GABA+ Fit Error (%)	5.04 (0.28)	3.82–6.90	5.19 (0.40)	2.74–8.42
FWHM (Hz)	19.17 (0.33)	16.26–20.80	19.19 (0.52)	16.94–24.47
SNR	13.52 (1.25)	6.42–20.84	13.95 (0.99)	8.21–21.88
PRESS				
Glx CRLB (%)	6.50 (0.20)	5–7	7.13 (0.26)	6–9
FWHM (Hz)	4.51 (0.15)	3.70–5.49	4.62 (0.11)	4.22–5.49
SNR	31 (0.74)	28–37	30.44 (1.21)	21–38

ASD, Autism Spectrum Disorder group; CTRL, Control group; S.E., Standard Error; FWHM, Full Width at Half Maximum; SNR, Signal to Noise Ratio; CRLB, Cramér-Rao Lower Bound. MEGA-PRESS and PRESS quality data were obtained from Gannet and LcModel softwares, respectively.

TABLE 3 | MRS voxel tissue proportions.

	ASD ($n = 14$)		CTRL ($n = 15$)	
	Mean (S.E.)	**Range**	**Mean (S.E.)**	**Range**
GM	0.33(0.005)	0.30–0.37	0.32 (0.005)	0.29–0.37
WM	0.61 (0.006)	0.57–0.66	0.62 (0.007)	0.58–0.66
CSF	0.07 (0.005)	0.03–0.11	0.06 (0.004)	0.04–0.09

ASD, Autism Spectrum Disorder group; CTRL, Control group; S.E., Standard Error; GM, Gray Matter; WM, White Matter; CSF, Cerebrospinal Fluid.

Both maximum MEP amplitude and curve slope of the input-output curve were found to be significantly correlated with GABA+ levels in the control group (max MEP: rs $= -0.665$, $p = 0.013$; curve slope: rs $= -0.692$, $p = 0.009$) but not in the ASD group (max MEP: rs $= -0.165$, $p = 0.590$; curve slope: rs $= -0.148$, $p = 0.629$) (**Figure 4**). The direct comparison

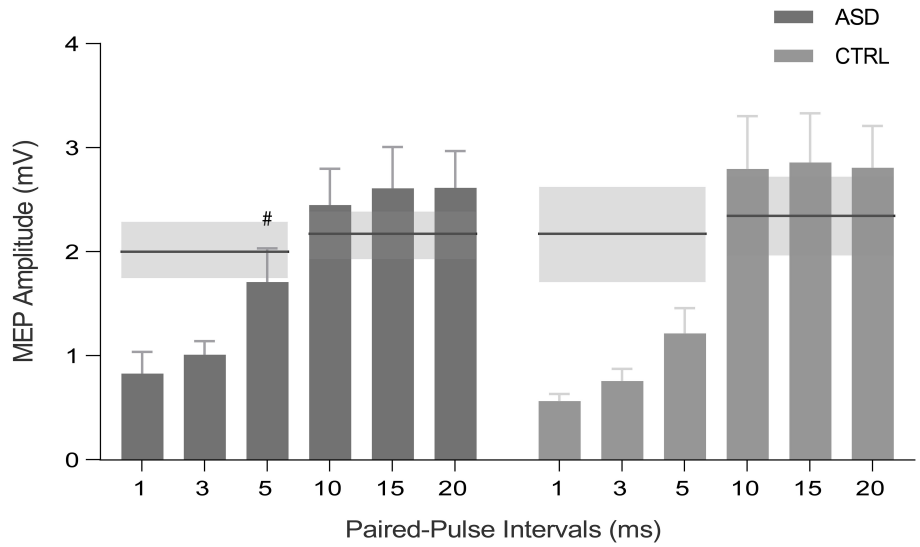

FIGURE 3 | MEP peak-to-peak amplitude for SICI (1, 3, and 5 ms) and ICF (10, 15, and 20 ms) intervals for ASD and control participants (CTRL). Horizontal lines represent the baseline condition (with no inhibitory or facilitatory effects), obtained by single-pulse TMS for each group and protocol. Inhibition occurs for bars below the horizontal lines, whereas excitation stands above the lines. SE of the means are illustrated in shaded horizontal bands and error bars. #$p > 0.05$ ASD, Autism Spectrum Disorder group; CTRL, control group; MEP, motor-evoked potential; SICI, short-interval intracortical inhibition; ICF, intracortical facilitation; S.E.; standard error of the mean.

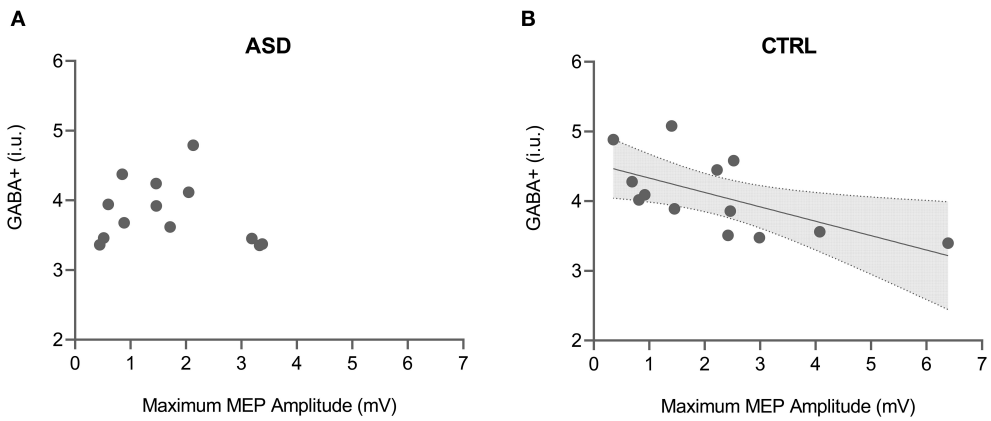

FIGURE 4 | Correlation between GABA+ levels and maximum MEP amplitude from input-output TMS protocol for both ASD **(A)** and control **(B)** groups. Shaded area in the scatter plot represents 95% CI for the significant correlation. ASD, Autism Spectrum Disorder group; CTRL, control group; GABA+, gamma-aminobutyric acid; MEP, motor-evoked potential.

of the slope of regression between groups revealed significant differences (max MEP: $p = 0.024$; curve slope: $p = 0.032$).

A significant correlation between the MEP amplitude ratio for SICI 1 ms interval and Glx levels was also found only in the control group (CTRL: rs = 0.636, $p = 0.035$; ASD: rs = −0.167, $p = 0.693$), even though the direct comparison between the regression slopes did not show significant differences ($p = 0.086$).

DISCUSSION

The current study aimed to comprehensively characterize GABAergic dysfunction in ASD from the neurochemical and physiological points of view, by obtaining measures from both MRS and TMS techniques.

Our findings show an increase of Glx levels in the motor cortex of individuals with ASD along with unchanged GABA+ levels. Moreover, we found a different pattern of inhibition in the ASD group for the SICI 5 ms interval.

Regarding MRS, evidence of enhanced Glx levels in ASD adults have been reported in different brain regions, such as the amygdala-hippocampal complex and auditory cortex (32, 33). In children, the same pattern was found in the anterior cingulate cortex and putamen (31, 68). These findings reinforced a long-standing hypothesis of increased excitation in ASD based on the observation of high comorbidity with seizure disorders, namely

epilepsy (4). So far, few studies assessed Glx levels in motor areas in ASD. In a recent study, Kolodny et al. (27) reported non-significant differences between adults with ASD and controls in a sensorimotor region which contrasts with our findings of significantly increased Glx levels in ASD. The fact that only a partial overlap exists in voxel placement in both studies may account for these differences since there is ample evidence for metabolite's levels to be region-dependent in both human (5, 6, 69, 70) and animal (71) studies.

In which concerns GABA+ quantification, the lack of significant differences between ASD and controls observed in our study is consistent with previous research exploring E-I imbalance in adults with ASD. Although some studies reported altered GABA+ levels toward both increase and decrease (28–30), others reported null results across a wide range of areas: dorsal lateral prefrontal cortex and basal ganglia (23, 72), medial prefrontal cortex (24, 25), occipital, auditory and parietal cortices (27) and left sensorimotor cortex and left ventral premotor cortex (28). Taken together, these findings seem to indicate that GABA+ levels are not consistently altered in ASD adults. This however, contrasts with the literature in children with ASD which, despite being more inconsistent, tends to report evidence toward a decrease in the levels of this inhibitory neurotransmitter (13, 14, 17). Porges et al. (73) posited, in a meta-analysis, that GABA levels rapidly increase during development, stabilize during early adulthood, and gradually decrease during adulthood and aging. We may speculate that, in ASD, GABAergic function develops more slowly during childhood until it reaches typical levels in adulthood.

Although it was not a primary objective of this study, we explored the link between MRS measures and autistic symptomatology assessed by ADOS and ADI-R diagnostic tools since there was previous evidence, from our group, of a negative association between GABA+ and communication and developmental delay ADI-R measures in children with ASD (20). In this work, we found that, in adults, Glx levels in the motor cortex were positively correlated with repetitive behaviors and interests, measured by ADI-R, and inversely correlated with social interaction score of ADOS. The interpretation of these correlations turns out to be challenging and should be taken with caution due to their exploratory nature, but it seems to indicate that perturbed E-I balance could, at some extent, help to explain the cognitive symptomatology in ASD.

In what concerns cortical excitability, as assessed by paired-pulse TMS protocols, an exploratory within-subject analyses showed the expected pattern of significant inhibition in all the studied SICI intervals for the controls. However, for the ASD group, we did not detect a significant inhibition for the SICI 5 ms interval. The fact that the paired-pulse intervals relate to different biological processes may explain the specificity of our findings. SICI is thought to be mediated by $GABA_A$ receptors which have been shown to exhibit decreased density in ASD as well as reduced protein expression for some $GABA_A$ receptor subunits (74, 75). Thus, we suggest that our observation is related to alterations in the cortical inhibition mediated by $GABA_A$ receptors. Alternatively, we may speculate that the transition between inhibitory (SICI) and facilitatory (ICF) intervals could be somewhat anticipated in ASD, along with the enhanced levels of excitatory Glx neurotransmitter reported in this study.

The fact that paired-pulse measures are similar between the ASD and the CTRL is in line with the work from Enticott et al. (37), who found no changes in cortical inhibition, assessed by SICI and LICI, in ASD, although showing SICI inhibition deficits in a subgroup of ASD patients who had language delay. However, in a previous study from the same authors, they reported reduced cortical inhibition (SICI) in high-functioning autism when compared to Asperger and typically developing groups (76), which may suggest that GABAergic dysfunction could be present in some ASD sub-groups characterized by specific phenotypic manifestations, including language delay (37). Although we selected a relatively homogeneous sample in what concerns to age range, intellectual functioning and diagnostic characterization, developmental acquisitions were variable in our ASD group. Given the high heterogeneity of the ASD phenotype, further stratified studies exploring different sub-groups could help to unravel the physiological specificities of E-I imbalance in this disorder. Oberman et al. (77) reported absence of changes in cortical excitability in ASD, measured by TMS, while pointing out a greater variability in the ASD group, with some participants exhibiting increased MEP amplitudes in the SICI and LICI inhibitory protocols. This agrees with our observation for the SICI 5 ms interval in the ASD group.

Regarding multimodal correlations, few studies reported associations between MRS and TMS, namely a relationship between GABA levels and SICI1ms and also slope of the TMS I-O curve (40, 41, 78). In our study, we found that GABA+ was negatively correlated with the maximum MEP amplitude and curve slope from the input-output protocol in the control group. This result was predictable from a physiological perspective since lower slope and MEP amplitudes may both be related to increased cortical inhibition (41) and refuted the opposite counterintuitive pattern observed by Stagg and colleagues (40). Remarkably, this correlation was not observed in the ASD group, as further demonstrated by direct comparisons, which suggests that the interaction between the neurochemistry and the neurophysiology underlying GABA transmission is distinct in autism. The link between these measures requires further investigation.

Further, we observed a correlation in the control group, showing that higher MEP amplitude ratio for SICI1 ms (less inhibition) was correlated with higher levels of Glx (more facilitation). To understand this relationship it is important to take into account that less inhibition can be equated with increased facilitation. Our result might therefore potentially reflect the relationship between inhibitory and excitatory mechanisms.

Here, we provided evidence supporting the E-I imbalance hypothesis in a group of adults with ASD. It is, however, important to take some limitations into consideration when interpreting our results. MRS technique provides an indirect measure of the GABAergic system which renders caution when drawing inferences from altered metabolite's quantifications. Additionally, a large voxel size was selected in order to maximize SNR leading to potential partial volumes effects (we

did nevertheless correct for voxel tissue composition). Moreover, we explored specifically the motor cortex which hampers the generalization of our findings to other brain regions. In the same line, to obtain a more homogeneous group, we selected only male high-functioning individuals with ASD which does not allow us to conclude about other specific sub-groups in the spectrum. Despite the great effort in recruitment, the implementation of rigorous criteria for data quality reduced the amount of eligible data for analyses giving them an exploratory nature that should be addressed in future confirmatory studies.

This study adopted a cutting-edge approach with the aim of probing E-I imbalance hypothesis in ASD by combining MRS and TMS measures. We gathered evidence that reinforces the notion of an altered balance between excitation and inhibition hypothesis driven by increased Glx levels in ASD. Moreover, our study gives important clues into the relevance of a multimodal approach allowing for direct comparison of neurochemical (GABA and Glx) and neurophysiological outcome measures related to inhibition, such as SICI5 ms and input-output curve parameters, in the autism spectrum disorder.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Comissão de Ética da Universidade de Coimbra. The patients/participants, or their parents when appropriate, provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

IB, IRV, and MC-B conceived the study. IB, AD, and MC-B made a substantial contribution to the study design, optimization of the protocol, and performed data analysis and interpretation. IB, AD, and RM collected data. IB wrote the first draft of the manuscript. All co-authors revised the work critically for important intellectual content and approved the manuscript in its final form.

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Identifying Age Based Maturation in the ERP Response to Faces in Children With Autism: Implications for Developing Biomarkers for Use in Clinical Trials

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Recent proposals have suggested the potential for neural biomarkers to improve clinical trial processes in neurodevelopmental conditions; however, few efforts have identified whether chronological age-based adjustments will be necessary (as used in standardized behavioral assessments). Event-related potentials (ERPs) demonstrate early differences in the processing of faces vs. objects in the visual processing system by 4 years of age and age-based improvement (decreases in latency) through adolescence. Additionally, face processing has been proposed to be related to social skills as well as autistic social-communication traits. While previous reports suggest delayed latency in individuals with autism spectrum disorder (ASD), extensive individual and age based heterogeneity exists. In this report, we utilize a sample of 252 children with ASD and 118 children with typical development (TD), to assess the N170 and P100 ERP component latencies (N170L and P100L, respectively), to upright faces, the face specificity effect (difference between face and object processing), and the inversion effect (difference between face upright and inverted processing) in relation to age. First, linear mixed models (LMMs) were fitted with fixed effect of age at testing and random effect of participant, using all available data points to characterize general age-based development in the TD and ASD groups. Second, LMM models using only the TD

group were used to calculate age-based residuals in both groups. The purpose of residualization was to assess how much variation in ASD participants could be accounted for by chronological age-related changes. Our data demonstrate that the N170L and P100L responses to upright faces appeared to follow a roughly linear relationship with age. In the ASD group, the distribution of the age-adjusted residual values suggest that ASD participants were more likely to demonstrate slower latencies than would be expected for a TD child of the same age, similar to what has been identified using unadjusted values. Lastly, using age-adjusted values for stratification, we found that children who demonstrated slowed age-adjusted N170L had lower verbal and non-verbal IQ and worse face memory. These data suggest that age must be considered in assessing the N170L and P100L response to upright faces as well, and these adjusted values may be used to stratify children within the autism spectrum.

Keywords: autism spectrum disorders, biomarkers, clinical trial methods, ERP, face processing, P100, N170, age

INTRODUCTION

Face processing is a foundational skill that supports social communication and has been proposed as a promising biomarker related to social function. Significant attention has been paid to the face processing skills of individuals with ASD, with group effects suggesting altered visual attention to facial features [e.g., (1)] and worse memory for faces [e.g., (2)]. While face processing delays have been proposed as a core feature in early ASD with negative developmental consequence on later social functioning (3, 4), significant heterogeneity exits including individual performance overlap with non-ASD groups [e.g., (5, 6); for discussion: (7)].

ERPs to Faces

The neural sources of face processing have been well-explored [e.g., (8–10)], with event-related potential (ERP) demonstrating early temporal differences in the processing of faces in the visual processing system. Bentin et al. (11) first identified the N170 component to faces as a negative-going voltage deflection recorded over the occipitotemporal scalp in adults; the N170 was greater in amplitude and faster in latency to faces than to other stimuli categories, and larger in amplitude but slower in latency to inverted compared to upright faces [A general review of the properties of the N170 in response to manipulation of the face is available *via*: (12)].

Children also display a developmental version of the N170, a negative ERP component that is similarly largest in amplitude at posterior temporal electrodes and larger in amplitude for eyes and upright faces in comparison to other non-face stimuli [e.g., (13–16)]. The N170 becomes markedly faster from 4 to 9 years with a less steep change between 10 and 15 years of age [e.g., Figure 7, (16)]. Mares et al. (17) suggest a lack of the adult-N170 inversion effect in childhood (6–11 years) (17). However, the N170 inversion effect [longer latency for inverted faces thought to index holistic processes (18)] was found in a sample of 8 to 9-year-old children when acquired during an explicit face recognition task but not until after 11 years in an implicit task. Thus, the inversion effect likely emerges during middle childhood and is vulnerable to tasks constraints. A further confound in developmental analyses is the presence of a *bifid* N170 peak present in 65% of children <12 years [Figure 6, (16)] or in children <9 years (14). Variability in waveform morphology and peak "peaking" protocols likely creates methodological inconsistencies that may further impact general interpretation of developmental trends.

In addition, the P100 is a positive deflection that is thought to index attentional processes, but also shows face sensitivity with more positive amplitude and shorter latencies to faces than other stimuli [e.g., (19, 20)], and faster latencies to upright compared to inverted faces (19, 21). In children, the inversion effect may be more prominent at the P100, with smaller amplitude but faster processing of upright compared to inverted faces (16). Less is known about the age-related changes in the P100 to faces despite it often being used to anchor the N170 window [e.g., (14)].

ERPs in Individuals With ASD

Both the N170 and the P100 have been found to differ between groups of individuals with ASD compared to typical development. Longer latencies for N170 responses to upright faces (suggesting slower processing) relative to neurotypical peers have been observed in ASD (22, 23). This slower N170 upright face latency effect has been found with ASD children aged 6–11 years in the ABC-CT sample (24) as well as 9–17-year-old children (25). Group differences have also been found in the P100 with slower latency to upright faces in children with ASD compared to children with TD [6–11 year old children: (24); 5–30 years: (26)].

A reduced inversion effect has been found in adults with ASD for P100 and N170 amplitude but not for the latency of either components (27). Similarly main effects of inversion but not group differences were found in samples that include children and adults [P100 latency (26)], and children aged 8–13 years [P100 latency: (28)]. In contrast, no inversion effects were found on N170 latency in three other developmental reports (25, 26, 28).

The N170 latency variability also appears to associate with social-emotional function in areas such as social competence, distress, empathy, emotional sensitivity, anxiety, introversion, shyness, and social withdrawal [for review: (23)] as well as face memory (24). Thus, while it is not clear that a reduced or slowed neural response is characteristic of individuals who reflect the broad autism spectrum, variability in this response may be related to aspects of the clinical phenotype of autism and other subdomains of social ability.

Developmental ERPs to Faces

While general age-based decreases in latency of ERP components have been reported, less is known about age-based trajectories in autistic individuals. Kang et al. note the significance of age as a moderator variable in their meta-analysis, with larger differences between ASD and TD in older youth and/or adult samples (23). In empirical reports, Neuhaus et al. found both the P100 and the N170 latency were significantly influenced by age, with the P100 showing an interaction between stimulus, orientation and age, and the N170 showing a main effect of age (26). Similarly, Hileman reported age effects for the P100 but not the N170, suggesting improved processing efficiency with age in attentional systems (25). In our ABC-CT sample, we found a significant relationship between age and the P100 (TD $r = -0.215$; ASD $r = -0.185$, $ps < 0.01$) and the N170 latency (TD $r = -0.369$; ASD $r = -0.350$, $ps < 0.01$), with faster latencies in older children (24). While it is clear there is age-based change in the ERP components related to face processing, precise metrics of growth, especially in ASD, have not been articulated.

Aims

If ERP markers are to be used as biomarkers in developmental populations, detailed analysis of chronological age-based maturation are necessary to evaluate whether or not stratification thresholds will required age-based adjustment (as used in many standardized assessments). The primary aim of this analysis is to characterize the age-based changes in neural systems represented by the N170 and the P100 *latency* response to face processing in the ABC-CT sample of 280 children with ASD compared to 119 children with TD. We focus on latency given that peak amplitude is impacted by trial-to-trial latency variability and previous work suggests a reliable decrease in peak processing time in childhood. As a secondary aim, we investigate the age-based characterization of N170 latency face specificity effect (FSE), which provides a metric of the neural specialization to faces by calculating the difference between the processing of upright faces compared to upright objects. We also include the inversion effect (IE) (upright face compared to inverted face). Third, we use age-adjusted N170 and P100 responses to identify whether these biomarkers can be used for stratification.

METHODS

Protocol

Details about the ABC-CT (Autism Biomarkers Consortium for Clinical Trials) protocol are published (29, 30). Data are available via (NDA study #2288) and the project is listed in ClinicalTrials.Gov (NCT02996669).

Briefly, the first phase of the ABC-CT included children aged 6.0–11.5 years, with 280 children with ASD (meeting gold standard ASD diagnostic criteria and full-scale IQ between 60 and 150) and 119 typically developing children (confirmed to be free of elevated psychiatric, psychological, or developmental concerns and full-scale IQ between 80 and 150) assessed at 5 sites using clinician, caregiver, and lab-based measures of social functioning and a battery of EEG and eye-tracking (ET) tasks. The protocol was administered during 2-day visits, with the EEG on the second day. Participants provided data at three timepoints (T1, T2 +6 weeks, and T3 +6 months). Distance in days between timepoint is provided in the Section Protocol, (**Supplementary Table 1**). Using a central IRB, informed consent/assent was obtained from guardians/participants after all procedures had been fully explained and the opportunity to ask questions was offered.

Participant Characteristics

Table 1 provides demographic characteristics of the enrolled ABC-CT sample at Time 1 ("All"), for the subsample contributing valid data to the analysis ("Faces"), and for the subsample not contributing valid data to the analysis ("No data"). Clinical observation was provided through use of the Differential Abilities Scale (DAS: Full Scale IQ, Verbal IQ, Non-Verbal IQ), Autism Diagnostic Observation Schedule-2 [Comparison Score (CS)], and the NEPSY Face Memory task. Parents provided additional characterization through the Vineland Adaptive Behavior Scales-3 (VAB3) interview (Socialization Standard Score, Communication Standard Score), and parent report questionnaires including the Social Responsiveness Scale [SRS Social Communication and Interaction T-Score (SCI) and Restrictive Interest and Repetitive Behavior T-Score (RIRB)], and Pervasive Developmental Disorder and Behavior Inventory [PDDBI Social Approach T-Score (SocApp)]. Further participant characterization is available in Faja et al. (31). In this report, age was based on the day of the EEG visit. Age did not differ between the ASD and TD groups when considering the total sample at Time 1 ($F = 0.045$, $p = 0.833$), nor when including only those with ERP data for the Faces assay ($F = 1.623$, $p = 0.20$).

EEG Acquisition

All procedures for standardization are available via request and in Webb et al. (30). All sites had an EGI 128 channel acquisition system, with either Net Amps 300 ($n = 3$) or 400 ($n = 2$), 128 electrode EGI HydroCel Geodesic Sensor Nets, Logitech Z320 Speakers, Cedrus StimTracker (for visual presentation timing), and a Dell 23″ monitor. A standard acquisition setup was implemented: 1,000 Hz sampling rate, 0.1–200 Hz filter, EGI MFF file format, onset recording of amplifier and impedance calibrations, and a 0.1 Hz digital high pass filter post-acquisition. EPrime 2.0 was used for experimental control.

The ERP Faces Assay included 6 blocks of 36 stimuli, comprised of 3 neutral female faces presented upright (FaceUp) or inverted (FaceInv) (32) and 3 houses presented upright (HouseUp) with visual angle was 12.3 × 9.3 degrees. The

TABLE 1 | Summary of participant characteristics at Time 1.

Time 1	ASD All	TD All	ASD Faces	TD Faces	ASD No data	TD No data
N total	280	119	252	118	28	1
N female	65	36	60	36	5	0
% female	23%	30%	24%	31%	18%	0%
Age in yrs	8.6 (1.6)	8.5 (1.6)	8.7 (1.6)	8.5 (1.6)	7.4 (1.4)	NA
DAS Verbal IQ	96.0 (20.7)	116.3 (11.2)	97.7 (19.8)	116.4 (11.2)	80.5 (22.1)	99 (NA)
DAS NonV IQ	97.5 (16.9)	112.2 (14.1)	98.7 (16.7)	112.4 (13.8)	87.3 (15.5)	81 (NA)
DAS Full IQ	96.6 (18.1)	115.1 (12.6)	98.1 (17.7)	115.4 (12.3)	83.0 (16.4)	86 (NA)
ADOS CSS	7.6 (1.8)	1.6 (0.9)	7.6 (1.8)	1.6 (0.9)	8.0 (1.3)	1 (NA)
VABS3 Soc SS	69.9 (16.1)	104.6 (9.2)	70.6 (15.9)	104.6 (9.2)	63.5 (17.5)	NA
VABS3 Com SS	76.4 (15.1)	103.4 (9.2)	77.3 (14.7)	103.4 (9.2)	68.6 (16.5)	NA
SRS-2 SCI T	72.7 (10.8)	42.5 (5.1)	72.4 (11.0)	42.5 (5.1)	75.2 (8.2)	38 (NA)
SRS-2 RIRB T	73.7 (12.2)	44.0 (3.7)	73.4 (12.4)	44.0 (3.7)	76.4 (9.4)	43 (NA)
PDDBI SocApp T	54.2 (9.3)	69.8 (3.0)	54.4 (9.4)	69.9 (3.0)	52.4 (8.4)	65 (NA)
NEPSY face memory SS	7.9 (3.7)	10.5 (3.5)	8.1 (3.7)	10.6 (3.5)	5.9 (3.0)	8 (NA)

Mean and standard deviation are presented for assessments for the full sample (All), the subset of participants providing at least one valid data point contributing to the analyses (Faces), and the subset of participants providing no valid data points (No data).

DAS, Differential Ability Scale; NonV, Non Verbal; ADOS, Autism Diagnostic Observation Schedule; CSS, Calibration Severity Score; VAB3, Vineland Adaptive Behavior Scales-3; Soc, Socialization; Com, Communication; SS, Standard Score; SRS, Social Responsiveness Scale; RIRB, Restricted Interests and Repetitive Behavior subdomain; SCI, Social Communication and Interaction subdomain; T, T-Score; PDDBI, Pervasive Developmental Disorder Behavioral Inventory; SocApp, Social Approach Behaviors Domain.

experiment included 216 trials, acquired in 6 blocks of 36, resulting in 72 trials per condition. Each trial consisted of a fixation crosshair (500–650 ms), stimulus (500 ms), and blank screen (500–650 ms). A schematic of the assay is presented in **Supplementary Figure 1**. During acquisition, the experimenter coded the participant's behavior for attention and compliance and any trial in which the participant did not attend to the image was discarded.

EEG Processing

Post-acquisition, EEG data was processed using the PREP algorithm (33) to remove line-noise, re-reference to a robust average reference, and interpolate bad channels relative to this reference. We then bandpass filtered the EEG at 0.1–30 Hz. Trials were segmented to 200 ms before and 500 ms after stimulus onset and unattended trials were removed. Baseline correction was applied using the 200 ms pre-stimulus interval. Artifact detection was done using the ERPLab function *pop_artextval* (34) with a threshold of 150 μV and a time window of −200 to 500 ms.

A participant's data was included if they had ≥21 artifact free and attended FaceUp trials. We focused on the right posterior-temporal region (RPT) for both components, which was created by averaging 5 channels (89, 90, 91, 95, 96); the analysis of lateral leads for the P100 (60–200 ms) and N170 (120–400 ms) and is consistent with prior publications [e.g., (16, 25, 26, 35, 36). The net layout is presented in **Supplementary Figure 2**. Trials were averaged by stimulus condition (FaceUp, FaceInv, HouseUp). The P100 Latency (P100L, 60–200 ms) and N170 Latency (N170L, 120–400 ms) peak amplitude and latency were identified using an automated algorithm and then visually inspected and adjusted at the individual level for the region of interest *via* manualized definitions available in Section EEG Processing. **Supplementary Figure 3** depicts the grand average waveforms. **Supplementary Tables 2, 3** provides information on missing data by group, timepoint, and variable of interest. Average number of trials (artifact free, attended) are presented in **Supplementary Table 4**.

Analytic Plan

For Aim 1, primary dependent variables were the N170L and P100L responses to FaceUp. For Aim 2, we also examined: (1) the face specificity effect for both components (FSE), which is the difference in the peak latency value for upright faces minus upright houses and (2) the inversion effect (IE), which is the difference in peak latency for upright faces minus inverted faces.

Age Based Development

Linear mixed models (LMM) were constructed for each relevant ERP component using all available data. To determine the variance structure in the ASD and TD groups, separate models were constructed in each group before the combined model was constructed. Final models had the fixed effects of timepoint, group, and mean-centered age at acquisition, and participant level random intercepts with different random effect variance structures for TD and ASD. Confidence intervals for fixed effects were estimated using likelihood profiles. Timepoint was included as a factor in the model to adjust for potential exposure effects of repeat testing. Random slopes were not included in the model because each participant only had, at most, three timepoints, two of which (T1 and T2) were close in time.

More complex models were also considered in the characterization of age-based changes in N170L and P100L in response to FaceUp. Models were compared using Akaike Information Criterion (AIC) values, with lower AIC values suggesting better model fit. For both FaceUp N170L and P100L, the models described above had a lower AIC than models with

the same random and fixed effects but with the addition of a fixed interaction effect between group and age at testing. Adding a quadratic term for age at testing to the models described above also resulted in models with higher AIC values for both models. AIC values for the three types of models that were assessed are available in **Supplementary Table 5**.

Age Adjustment

The purpose of residualization using the TD group is to understand age-based distributional characteristics of the ASD sample independent of developmental or maturational expectations. That is, the residualization is derived based on the developmental trajectory in the TD group. Hence it can be used to investigate whether the latencies in the ASD sample are slower or faster than what they would be expected to be based on age. Even though they are derived based on age-specific comparisons, the residulization values are age-free.

To create these values, random intercept models with fixed effect of age at testing and random effect of participant were fitted using all available data points in the TD group and then used to calculate age-based residuals in the TD and ASD groups. A positive residual value in the ASD sample indicates that the latency was slower than would be expected in a typically developing child of the same age. A negative residual value indicates that the latency was faster than would be expected in a typically developing child of the same age. See **Figure 1** for the relation between the (raw) N170L FaceUp values and the age-adjusted or residualized N170L (aN170L) FaceUp values. Raw values for the N170L and P100L for the FaceUp are presented in **Table 2**. Raw values for FSE and IE are presented in **Supplementary Tables 6, 10**; ICC values are presented in **Supplementary Table 7** and adjusted values are presented in **Supplementary Table 11**.

Stratification

We then investigated the potential use of these age-adjusted ERP values for stratification. That is, if distributions of the age-adjusted residuals contain a tail, this may represent distinct neural subgroups of children with ASD. To this end, a cutoff point for each adjusted-ERP component was calculated based on the upper 10% of all available age-adjusted residual values from the TD group. We identified the participants in the ASD group whose T1 values were greater than or less than that age-adjusted cutoff. We compared clinical characterization for autistic children stratified by the cutpoint using unadjusted ANOVAs.

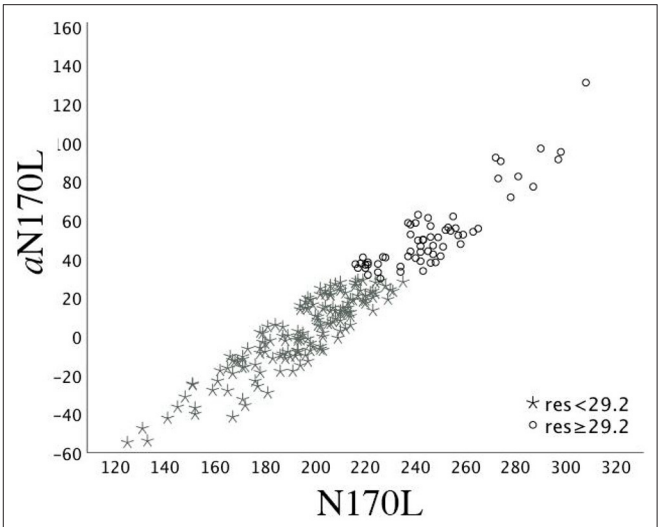

FIGURE 1 | Time 1 N170L and *a*N170L for the ASD group. Relation between the raw Time 1 N170L (x-axis) and the age-adjusted N170L (aN170L)(y-axis) in response to upright faces. Children with ASD and slowed age-adjusted N170s are identified by black open circles; Children with ASD and standard age-adjusted N170s are identified by gray stars. The use of the residual cutpoint of >29.2 reflects the ASD sample overlap with 10% of the TD group.

RESULT

Age Based Development Using Linear Mixed Models for Upright Faces

Based on preliminary exploration of P100L and N170L, it was determined that there were substantial differences in variance structure between the ASD and TD groups. Models were therefore constructed with differing random effect variance structure for ASD and TD groups when data complexity allowed. **Table 3** shows fixed and random effects for the model. It should be noted that *p*-values have not been included in descriptions of fitted LMMs; this is a deliberate omission (37).

The TD and ASD participant variances, estimate, on average, how much the ERP latency component deviates from their age- and timepoint-based predictions across participants, specific to each diagnostic group. Since age at testing has been mean-centered (with sample mean 8.7 years old), the fixed effect intercept represents an estimate of mean ERP component score for typically developing individuals at baseline.

For FaceUp, the average N170L was an estimated 0.018 ms (95% CI: −0.022, −0.013) faster for each additional day of age (**Table 3**, left) or 6.57 ms per year. Between-participant variance for N170L FaceUp is greater in the ASD group ($SD_{ASD} = 25.4$) than the TD group ($SD_{TD} = 19.9$).

The average P100 Latency FaceUp was an estimated 0.0056 ms (95% CI: −0.079, −0.0033) faster for each additional day of age (**Table 3**, right) or 2.05 ms per year, adjusting for group and timepoint. Between-participant variance for P100L FaceUp is greater in the ASD group ($SD_{ASD} = 13.7$) than the TD group ($SD_{TD} = 9.7$).

Age-Adjusted Residuals for Upright Faces

Across N170L (**Figure 2**) and P100L (**Figure 3**), the distribution of residuals in the ASD group (**Figures 2F**, **3F**) suggest slower than expected N170 latencies in the ASD group (**Figure 2C**) similar to what we reported for raw values (24).

Overall, there was greater variability in ERP component scores observed between individuals in the ASD group than

TABLE 2 | Summary statistics for raw and residualized N170L and P100L in response to upright faces.

	FaceUp N170L		FaceUp *a*N170L		FaceUp P100L		FaceUp *a*P100L	
	TD	**ASD**	**TD**	**ASD**	**TD**	**ASD**	**TD**	**ASD**
N	336	624	336	624	336	623	336	623
Missing	21	216	21	96	21	217	21	97
Mean	193.60	206.23	0.19	14.01	117.57	121.74	0.17	4.76
Median	193.0	203.5	−1.82	11.45	117.0	119.0	−0.38	1.95
SD	27.13	34.16	25.04	32.31	13.12	16.89	12.60	16.76
Skewness	0.33	0.92	0.47	1.14	0.96	1.20	0.86	1.23
SE of Skewness	0.13	0.010	0.13	0.10	0.13	0.10	0.13	0.10
Kurtosis	0.26	2.97	0.93	3.54	4.35	2.07	4.51	2.16
SE of Kurtosis	0.27	0.20	0.27	0.20	0.27	0.20	0.27	0.20
Min	125.0	125.0	−68.0	−60.0	82.0	82.0	−41.0	−32.0
Max	276.0	393.0	88.0	185.0	189.0	192.0	69.0	72.0
% 10	158.7	166.5	−28.61	−23.21	103.7	104.0	−13.27	−12.61
% 25	177.0	186.0	−15.51	−7.15	110.0	111.0	−6.85	−5.24
% 30	181.0	190.0	−13.82	−2.60	112.0	113.0	−5.53	−3.83
% 50	193.0	203.5	−1.82	11.45	117.0	119.0	−0.38	1.95
% 70	204.9	219.0	12.75	26.01	122.0	126.0	5.18	8.77
% 75	208.0	224.0	16.90	30.97	124.0	128.0	6.77	10.41
% 90	229.30	246.0	29.20	50.68	130.30	145.0	12.57	27.28

The model used to calculate residuals was a mixed effect model fitted in the TD group. Age was a fixed effect and participant ID was a random effect. Skewness is close to 0 when the distribution is symmetrical, negative when the left tail of the distribution is longer, and positive when the right tail of the distribution is longer. Larger values of kurtosis indicate heavier tails (kurtosis = 3 for a univariate normal distribution).

TABLE 3 | Random and fixed effects for N170 latency and P100 latency to upright faces.

N170 Latency FaceUp			**P100 Latency FaceUp**		
960 observations, 370 participants			**959 observations, 369 participants**		
Fixed effects	**Estimate (95% CI)**	***t*-value**	**Fixed effects**	**Estimate (95% CI)**	***t*-value**
Intercept	195.4 (191.0, 199.9)	85.9	Intercept	116.8 (114.6, 119.0)	105
Age	−0.018 (−0.022, −0.013)	−7.8	Age	−0.0056 (−0.0079, −0.0033)	−4.7
ASD group	14.4 (9.0, 19.8)	5.2	ASD group	4.61 (1.9, 7.3)	3.3
T2	−3.4 (−6.3, −0.43)	−2.2	T2	0.78 (−0.67, 2.2)	1.1
T3	−3.3 (−6.3, −0.18)	−2.1	T3	0.95 (−0.57, 2.5)	1.2
Random effects	**Variance (SD)**		**Random Effects**	**Variance (SD)**	
TD	396.4 (19.9)		TD	93.8 (9.7)	
ASD	645.6 (25.4)		ASD	187.0 (13.7)	
Residual	350.0 (18.7)		Residual	84.9 (9.2)	

Mixed effect model fits a random y-intercept with differing variances for typically developing (TD) and autism spectrum disorder (ASD) groups.

between individuals in the TD group. Higher between-participant variation is evidence for greater heterogeneity in the FaceUp N170L and P100L components across the ASD participants. This pattern of greater between-participant variability in the ASD group is consistent across all ERP component models.

FSE and IE

We provide results for the FSE and IE analyses in Section "SM 3.3 FSE and IE Results" (**Supplementary Tables 6–9**) as the raw values showed poor test-retest stability over the 6 week period.

Use for Stratification

For Aim 3, we possible that these markers, specifically the *a*N170L and *a*P100L to upright faces, may be useful in identifying a more homogeneous subgroup within children with ASD. We suggest a cutoff point based on the upper 10% from the TD group based on all available age-adjusted residual values (*a*N170L; **Table 2**, TD %90 = 29.20); this resulted in a subgroup of 62 ASD participants (29%) at T1 with slowed peak values for their age. For comparison, the *a*N170L for the age-adjusted slowed group (when *a*N170L > 29.2) had a mean (raw) N170L of 247.21 ms (SD 21.38. range 216–308) in contrast to the standard group (*a*N170L ≤ 29.2), which had a mean N170L of 194.30 ms (SD =

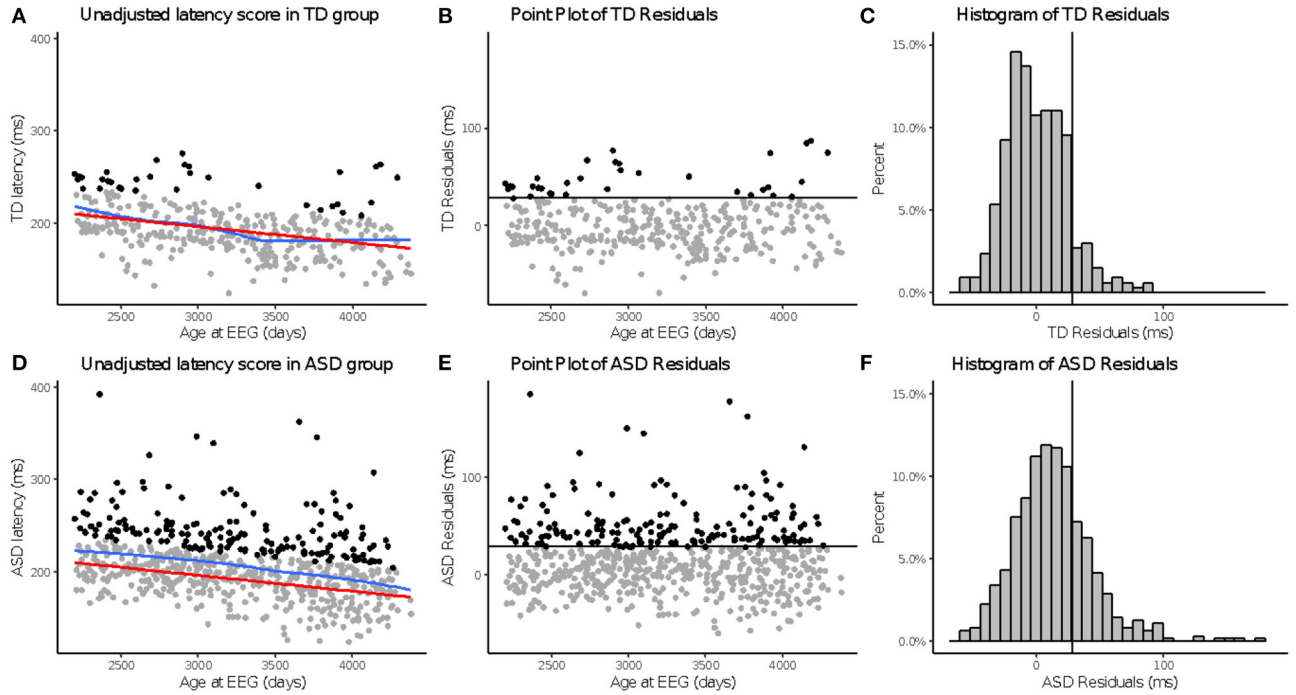

FIGURE 2 | *a*N170 latency to upright faces. Age-adjusted N170L in response to upright faces. Graphs depict the TD group in the top row **(A–C)** and ASD in the bottom row **(D–F)**. **(A,D)** Red line indicates predicted values of N170L based on the fitted model, while the blue line indicates the locally estimated scatterplot smoothing (LOESS) for each group. **(B,E)** Residual values calculated using the fitted model. **(C,F)** Black line indicates a cutoff point derived from the upper 10% of all age-adjusted N170L scores in the TD group. Data points greater than that cutoff point are black **(A,B,D,E)**.

21.92, range 125–235). Those children with ASD and a slowed *a*N170L compared with those with ASD and a standard *a*N170L were of a similar age ($F_{1,212} = 1.07$, $p = 0.30$) but had lower Full Scale IQ, Verbal IQ, and NonVerbal IQ ($F_{1,212} > 7.21$, $ps < 0.01$), and Face Memory SS ($F_{1,212} = 4.97$, $p = 0.03$). There were no differences between the ASD groups on measures of autism traits (ADOS CSS $F_{1,212} = 3.04$, $p = 0.08$; SRS SCI $F_{1,209} = 0.36$, $p = 0.53$; SRS RIRB $F_{1,210} = 0.45$, $p = 0.50$; PDDBI SocApp $F_{1,204} = 0.40$, $p = 0.53$) or adaptive skills (Vineland Socialization $F_{1,211} = 0.030$, $p = 0.86$, Communication $F_{1,211} = 0.75$, $p = 0.39$).

Using a similar strategy, for the P100L, a subgroup of 10% of TD participants with the slowest *a*P100L values (**Table 2**, TD %90 = 12.57) identified a subgroup of 49 ASD participants (23%) at T1 with slowed response for their age. For comparison, the raw P100L for the age-adjusted slowed group (when *a*P100L > 12.56) has a mean P100L response of 145.57 ms (SD 14.06 range 126–190); in contrast to the standard group (when *a*P100L ≤ 12.56) who had a mean P100L response of 114.49 ms (SD 9.92 range 90–135). Those children with ASD and a slowed *a*P100L compared with those with ASD and a standard *a*P100L were of a similar age ($F_{1,212} = 0.312$, $p = 0.58$); the stratification did not result in ASD subsamples differing on any of the behavioral measures included in this report such as the cognitive characterization measures ($F_{1,212} \leq 0.59$, $ps \geq 0.51$), measures of autism diagnosis or autistic behaviors ($F \leq 1.59$, $ps \geq 0.21$), or adaptive skills ($Fs < 0.68$, $ps > 0.41$).

DISCUSSION

Upright Face Response

The primary aim of this analysis was to characterize the N170 latency and P100 latency age-related response to *upright faces* in children with ASD in relation to typical chronological age-related changes (Aim 1). Overall, and consistent with previous reports, N170 latencies to upright faces were slower in younger children and in children with ASD. Specifically, N170 latency in response to upright faces was associated with age, and decreased in peak latency of 6.57 ms per year across our 6–11-year-old sample. We also found that the P100L to upright faces was associated with age, with a 2.04 ms decrease in latency per year. This data suggests that the effects of age must be considered in evaluating "slowed" N170L and P100L in response to upright faces, as even within a relatively narrow age, the processes underlying these components are improving (in terms of latency) at different rates in childhood. Slight differences in age distribution could influence the identification of group differences. Important for age-based adjustments, the linear relationship between age and upright face-related ERP components make it relatively straightforward to develop age-adjusted values.

Face Specificity Effect and Face Inversion Effects

We relegated the results for the FSE and the IE to **Supplemental Materials** due to the poor test-retest stability

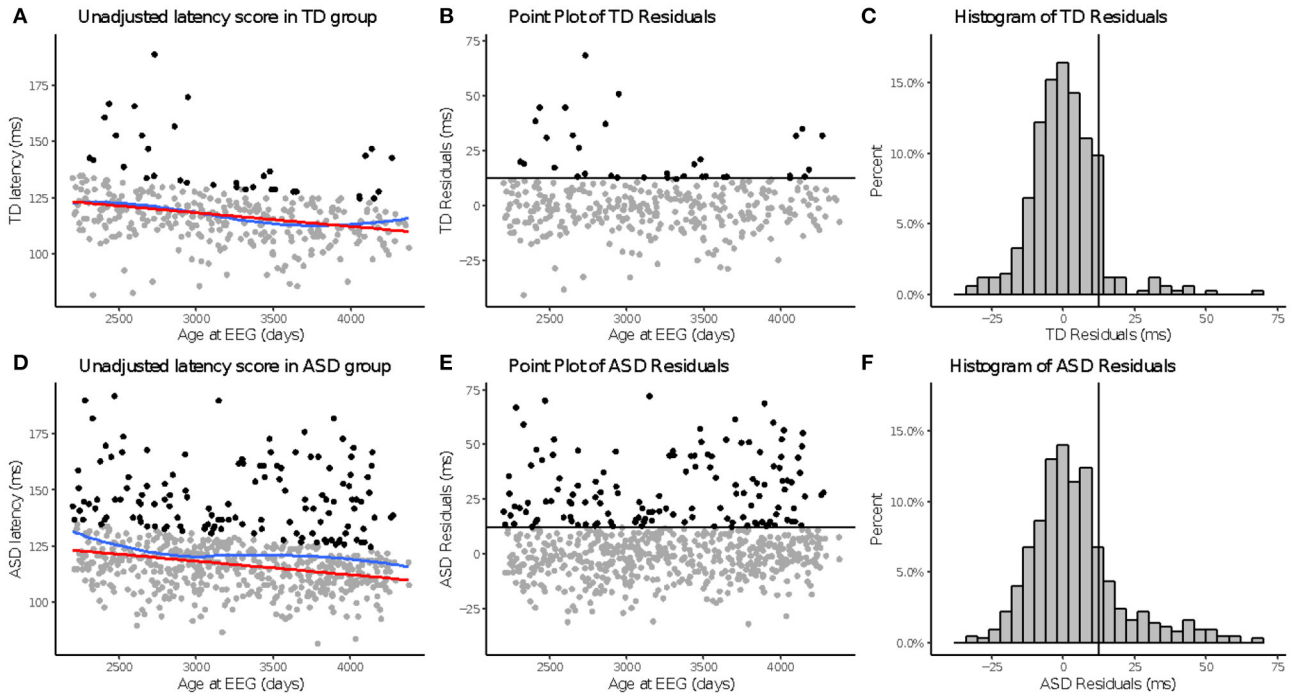

FIGURE 3 | *a*P100 latency to upright faces. Age-adjusted P100L in response to upright faces. Graphs depict the TD group in the top row **(A–C)** and ASD in the bottom row **(D–F)**. **(A,D)**: The red line in column 1 indicates predicted values of P100L based on the fitted model, while the blue line indicates the locally estimated scatterplot smoothing (LOESS) for each group. **(B,E)**: Graphs show the residuals values calculated using the fitted model. **(C,F)**: Black lines indicate a cutoff point derived from the upper 10% of all age-adjusted P100L scores in the TD group. Data points greater than that cutoff point are also in black **(A,B,D,E)**.

performance of these markers [e.g., ICC≤0.5, (38)]. This lack of stability makes these difference score markers less useful for clinical trials (in this sample). It is possible that the markers might show greater stability under a different protocol (e.g., shorter time interval). However, given these results and in comparison to the relatively higher stability values of single-condition ERP markers, we do not suggest incorporation of these into clinical trials without further evaluation.

In regard to face processing more broadly, it has been suggested that the early neural selectivity or preference for faces as a category of stimuli is present by 4–5 years, and while the responses to the FaceUp and FaceInv mature and shorten in latency with age (14, 39), the differential processing of upright faces compared to objects or inverted faces, at either the P100 or the N170, did not seem to undergo age based differential change in our age group. In our analyses, we utilized difference scores for both the FSE and the IE, with a negative response reflected a faster FaceUp response in comparison to the contrast stimuli (HouseUp and FaceInv, respectively). Unexpectedly, across our sample, only the responses for P100L demonstrated a "face upright" preference, with a negative difference score in this pattern with ≥90% of TD participants (P100L IE 90.1%; FSE 100%) and ≥79% of ASD participants (P100L IE 79.8%; FSE 83.8%) demonstrating faster latencies to FaceUp than the contrast stimuli (**Supplementary Table 6**).

In contrast, the N170L did not show consistent differentiation of FaceUp and the contrast stimuli. N170L IE and FSE were, on average positive, with only a minority of the sample having values in the negative range (IE < 0: TD 38%; ASD 36.8% FSE < 0: TD 42%; ASD 45%). This confirms prior reports of an inconsistent IE in this age range. Further, there was not a while a clear trend in the association between age and the primary ERP components to upright faces, as the associations between age and the FSE and IE were minimal (N170L FSE) or negligible (P100L FSE, N170L IE, P100L IE). It has been suggested that the early neural selectivity or preference for faces as a category of stimuli is present by 4–5 years, and while the responses to the FaceUp and FaceInv mature and shorten in latency with age (14, 39), the differential processing of upright faces compared to objects or inverted faces, at either the P100 or the N170, did not seem to undergo age based differential change based on our analyses.

Face Processing in ASD and Stratification

There are several potential uses for EEG biomarkers including stratification for inclusion, diagnosis or likelihood of developing ASD, prognostic, predictive, and surrogate endpoints (40). Both the N170 and P100 latency FaceUp biomarkers showed group discrimination, that is, with more positive or larger age-adjusted residual values in the ASD group compared to the than in the TD group; this response of slowed responses when adjusted for age, is similar to prior reports of slowed raw values [e.g., (23)]. However, there was significant overlap in the sample distributions, and it is unlikely that these ERP latency biomarkers would be useful for diagnostic purposes. As an alternative, we demonstrate that the

using the age-based adjusted N170L, we can identify a group of children who have lower scores in face memory, cognitive, and verbal ability.

Caveats

Of importance, if used as an inclusion variable, the biomarker would also need to consistently measure a trait of an individual. That is, if slowed face processing was a trait characteristic of a specific subgroup of autistic children, we would expect that this subgroup would be consistent across measurements. Our study was limited to having re-test values at +6 weeks, with stability influenced by trait, state, as well as measurement error. Our ICC values [N170L FaceUp $ASD_{T1-T2} = 0.662$; P100L FaceUp $ASD_{T1-T2} = 0.680$; (24)] suggest moderate stability over 6 weeks. Participants were not required to maintain stable treatments across the study and thus six-weeks may reflect a period of potential clinical change for some participants. Further evaluation of the usefulness of these ERP components as stratification variables at inclusion would benefit from assessment of reliability from periods more reflective of the time course of a clinical trial screening to baseline assessment period (e.g., $\leq$1 week).

This dataset, like many others in ASD research and clinical practice, included uneven distribution of sexes and the potential non-randomness of missing EEG data. Identifying whether or not age based adjustments also need to be sex or gender adjusted (as used in the SRS), will require inclusion of a larger sample of TD females. Further, while a sample of 60 autistic females is still higher than many other reports, there are substantial differences in the presentation of ASD between males and females and thus greater investigation of variability in ASD females is required.

In addition, missing data may not have been random. While we included 908–960 valid EEG data points (76–80% valid depending on the component and stimulus) making this one of the larger EEG datasets, there is more data loss than found in some other behavioral and experimental measures (e.g., eye tracking). For EEG, possible reasons for missing data at a given time point include ones that are common across measures (drop out), but also reflect the ways in which the child interacts with the experiment (boredom, behavioral non-compliance), and factors that may represent altered neural functioning (trial variability resulting in failure to show a peak response). As seen in **Table 1**, participants with no valid EEG data tended to have more impairing ASD symptoms, lower cognitive ability, and lower language ability. If symptom severity drives missingness of EEG data, EEG variables may have utility as a biomarker only within a certain range of symptom presentation.

Lastly, the biomarkers and behavioral/clinical assessments we include in this manuscript reflect a small selection of the potential variables that are available from the ABC-CT. Inclusion of alternative topographical responses (e.g., left hemisphere regions), amplitude of the ERP components or power of fundamental neural frequencies (e.g., alpha, theta), and measures of connectivity also need to be investigated in regard to their age based development. Further, exploration of these in relation to (novel) behavioral composites that may be more proximal to face attention will also be important. As the intent is to strengthen the toolbox of measures that can inform the clinical trial protocols, this manuscript represents a first step to identify if and how age based adjustments may be approached.

CONCLUSION

Using the large dataset from the ABC-CT, we identify age related development in the latency of the P100 and N170 to upright faces and suggest that age-based adjustments will be necessary for implementation of these biomarkers in clinical trials. Further, we identified a clinically more impacted subgroup when using age-adjusted N170L values, suggesting the importance of considering peak latency values relative to chronological age-expected values, rather than absolute cutpoints.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: Repository Data are available from NIMH NDA (#2288) (https://nda.nih.gov/edit_collection.html?id=2288).

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Yale Human Research Protection Program. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

SW, IE, CS, DS, AN, SF, AL, FS, and JM contributed to the conceptualization, analysis and drafting the work, and editing of the manuscript. All authors made substantial contributions to the conception or design of the work and read and provided review and approval for publication of the content. All authors contributed to the intellectual content and review and approval of the manuscript.

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Hutchins, Julie Holub, Minah Kim, Beibin Li, Samantha Major, Michael Mariscal, Samuel Marsan, Takumi McAllister, Adriana S. Méndez Leal, Lisa Nanamaker, Charles A. Nelson, Fleming Peck, Helen Seow, Laura Simone, Dylan Stahl, and Andrew Yuan. We refer to a number of experiments as well as support documents detailing our standard operation procedures and manuals of operation for the ABC-CT Feasibility phase and Main Study phase; these document can be accessed by request from the principal investigator (james.mcpartland@yale.edu) and additional project information can be found *via* our website.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpsyt.2022.841236/full#supplementary-material

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Distinct Patterns of Cognitive Outcome in Young Children With Autism Spectrum Disorder Receiving the Early Start Denver Model

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Evidence-based, early intervention significantly improves developmental outcome in young children with autism. Nonetheless, there is high interindividual heterogeneity in developmental trajectories during the therapy. It is established that starting intervention as early as possible results in better developmental outcomes. But except for younger age at start, there is no clear consensus about behavioral characteristics that could provide a reliable individual prediction of a child's developmental outcome after receiving an early intervention. In this study, we analyze developmental trajectories of preschoolers with autism who received 2 years of intervention using the Early Start Denver Model (ESDM) approach in Geneva, Switzerland in an individual setting (n = 55, aged 28.7 ± 5.1 months with a range of 15–42). Our aim was to identify early predictors of response to intervention. We applied a cluster analysis to distinguish between 3 groups based on their cognitive level at intake, and rates of cognitive change over the course of intervention. The first group of children only had a mild cognitive delay at intake and nearly no cognitive delay by the end of intervention (Higher Cognitive at baseline: HC). The children in the two other groups all presented with severe cognitive delay at baseline. However, they had two very different patterns of response to intervention. The majority significantly improved developmental scores over the course of intervention (Optimal Responders: OptR) whereas a minority of children showed only modest improvement (Minimal Responders: MinR). Further analyses showed that children who ended up having an optimal 2-year intervention outcome (OptR) were characterized by higher adaptive functioning at baseline combined with rapid developmental improvement during the first 6 months of intervention. Inversely, less significant progress by the sixth month of intervention was associated with a less optimal response to treatment (MinR).

Keywords: autism spectrum disorders, early intervention, predictors, response to treatment, heterogeneity, minimal responder

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INTRODUCTION

Autism spectrum disorder (ASD) is characterized by impairments in communication and social interactions, along with restricted and repetitive behaviors (1). Over the last three decades, several comprehensive, evidence-based early intervention (EI) approaches have been developed for young children with ASD, with the aim to improve their social communication, cognitive functioning,

and adaptive skills (2–7). The principles of EI usually comprise a significant number of hours (usually more than 15 h per week) as well as an early age of onset (usually younger than 4 or 5 years old) (8). Systematic reviews and meta-analyses showed positive effects of EI on cognition, adaptive skills and communication at the group level (9, 10). Nevertheless, many EI studies have reported a relatively heterogeneous response to these interventions, where most children show significant improvements, while others make smaller gains (11, 12). Despite important efforts to better understand variables affecting treatment response, it is currently not possible to predict to what extent a child will respond to intervention based on his or her behavioral characteristics at intake (13). In the current therapeutic context and in the absence of additional knowledge about individual predictors of outcome, many authors suggest that intensive early intervention should be an intervention of choice for young children diagnosed with ASD (14, 15) regardless of their specific behavioral or symptom profile. Yet, in the global framework of precision medicine (16), there is an urge to develop more individualized guidelines for intervention in ASD. Given the importance of providing effective programs for children with ASD as early as possible, and because of the costs and parental investment associated with early intervention, it is crucial that we move away from a "one size fits all" service provision model, and find ways to tailor a child's intervention to their specific needs, choosing therapy approaches based on the child's individual profile at diagnosis (17–19).

During the last decade, Early Start Denver Model (ESDM) has emerged as a promising Naturalistic Developmental Behavioral Interventions (NDBI) (7). NDBIs represent a category within the broader context of EIs, as discussed by Vivanti and Stahmer (20). Briefly, NDBIs designates approaches that integrate the methods derived from behavioral learning and developmental science. Main principles include varying the stimuli for learning, using the activities the child enjoys the most and emphasis put on developmental prerequisites. Within NDBIs, ESDM is notably characterized by its overall effectiveness, its emphasis on natural environment teaching, comprehensive learning objectives and parental involvement. ESDM intervention has originally been implemented in an individualized setting (one therapist for one child, I-ESDM), but other applications have been developed such as G-ESDM where one therapist works with a little group of children and P-ESDM where parents/caregivers actually provide the intervention under supervision. In their 2010 landmark randomized controlled trial (RCT) (2), Rogers and Dawson reported a mean increase of 18 IQ points in a sample of 24 toddlers with ASD receiving the I-ESDM intervention over 2 years. Numerous studies have replicated these results [for a review see (10)], highlighted a good reproducibility in different contexts such as the European one (21–23) and demonstrated the cost-effectiveness of ESDM intervention (17, 18). Overall, the ESDM approach has been shown to significantly increase cognitive, communication and adaptive skills at the group level (24). However, the inter-individual variability in child response to treatment (RTT) is high, as with all types of EI (25). To date, research about RTT in ESDM remains sparse and most studies focusing on homogeneous and individualized therapy settings comprised limited sample size. Younger age at start has emerged as an important moderator of optimal outcome, probably due to higher brain plasticity (26, 27). Age left aside, there are no behavioral characteristics child that are recommended by any international guidelines as a reliable individual predictor of RTT in ESDM, despite many attempts to identify some (14, 28). For instance, Vivanti et al. (29) attempted to identify predictors of RTT in children receiving G-ESDM intervention. Their study showed that developmental gains after one year of treatment were best predicted by higher imitation skills, goal understanding, and more advanced skills in the functional use of objects at baseline. This study offered insight into how children with certain baseline competencies might progress faster in a G-ESDM setting. However, outcomes were assessed after only 1 year of intervention and baseline measures used in this study were based on original tasks (i.e., specially developed for this study and not available in the common practice), making its results poorly reproducible. In addition, its group setting makes its conclusion hardly generalizable to the canonical individualized setting of ESDM. Besides, some authors identified that lower cognitive level at baseline could be related to higher RTT, although this effect could be biased by a larger potential for gain in children with very low cognitive profile (30, 31). This brief review shows that various behavioral characteristics (e.g., global cognitive level or imitation skills) at baseline modulate the outcome of an ESDM intervention. Nevertheless, none of these parameters has reached the status of being a reliable predictor of individual response to ESDM intervention recommended by international guidelines yet (14). It is therefore currently not possible to know to which extent the ESDM intervention will be effective when advising it. Yet, the identification of characteristics that promote the response to a specific intervention could in the future be of great help to the clinical practice when referring a child to one EI or another. Similarly, new approaches or goals could also be implemented to promote the emergence of these predictors to create cascading effects on children's intervention response. Great interindividual heterogeneity in response to intervention has been identified as a major limitation to this quest (13). A promising way of dealing with this heterogeneity relies on moving from a whole-group approach to the identification of distinct subgroups exhibiting specific patterns of response to intervention (32).

In the present study, we aim to identify early children's behavioral characteristics that could serve as predictors of outcome after receiving a specific and homogeneous NDBI (here, I-ESDM). To do so, we explored the developmental trajectories of 55 preschoolers with ASD who completed 2 years of individualized and intensive (20 h per week) ESDM intervention available in Geneva, Switzerland. We used a longitudinal single group design without a control population, similar to previous studies in the field (25, 26, 29). Indeed, because of ethical as well as logistic considerations, a random referencing to either the ESDM intervention program or any other community treatment was not achievable. We first investigated if our sample's outcome data, in terms of cognition, symptom severity and adaptive functioning, reflected findings described in the ESDM literature. We then parsed the heterogeneity in our sample's outcome by using cluster analysis (CA) and cognitive scores as the main

outcome measure. CA highlighted three different groups based on cognitive outcome. We further explored baseline differences as well as early rates of change between the three groups to identify potential predictors of 2 year treatment outcome.

METHODS

Participants

Our original sample included 61 participants who completed 2 years of ESDM intervention in Geneva, Switzerland. Five participants were not included in the analyses because of missing data regarding their developmental assessment at baseline and one participant because of missing data at the end of the intervention. Missing evaluations were all caused by logistical issues (e.g., evaluation material not available at this time) and not because of children characteristics (e.g., invalid evaluation because of the child's behavior). Full description of the six excluded children is provided in **Supplementary Table 3**. There was no significant difference between the excluded participants and the final sample. Our analyses were thus based on the data collected from 55 participants (see **Table 1**).

There was no exclusion criteria based on co-occurring somatic, neurologic, or genetic disorder, as long as they were not affecting the validity of behavioral measures (e.g., major cerebral palsy). There was no systematic genetic or neurological screening done in our protocol. Genetic, somatic and neurologic diagnosis were screened with parental questionnaires. To our knowledge, no children were affected by any neurologic condition (e.g., epilepsy) diagnosed by a neurologist following active consultation by parents. No parents reported any diagnosis of major somatic disorders that could have affected the validity of behavioral measures. Twenty-four participants' parents reported having met a clinical geneticist. Four of them reported a genetic finding that could be "causative for ASD" according to the geneticist's report.

All children were referred to the intervention program after receiving a clinical diagnosis of ASD according to Diagnostic and Statistical Manual of mental disorders, 5th edition (1) criteria and Autism Diagnosis Observation Schedule-Generic (ADOS-G) (33) or 2nd edition (ADOS-2) (34) diagnosis cut-offs. For children that were administered the ADOS-2 Toddler module (which does not provide a diagnostic cut-off) at baseline, the "mild to moderate concern for ASD" cut-off had to be overreached. All children assessed with a Toddler module at baseline met the diagnosis cut-off (using ADOS-2 module 1 or 2) on their visit 1 year later, even though this was not an inclusion criterion for our study.

Enrollment in the intervention program was also conditioned by an age criterion: participants had to be able to participate for two full years prior to age of school entry. In Geneva, a child has to be 4 years old by July 31st to enter school in August of the same year. In our sample, one child was too old (42 months old at baseline) to meet this criterion but was still enrolled in the program as there was an available position. This results in a sample that is fairly homogeneous in age at start (28.7 ± 5.1 months, see **Table 1**). At least one parent had to be fluent in either French or English. Therapists fluent in both these languages were available to provide intervention, follow-up and parental coaching. The latest census in Geneva (35) reports that 92.3% of the population use either French or English as a first language. We must add to this percentage the people fluent in French or English as a second language. Thus, the vast majority of the population in Geneva was eligible for the intervention program based on the language inclusion criterion. Besides, there has been increasing concerns about socio-economic representativeness of the samples used in EI research (36, 37). To date the majority of ESDM studies are based on a white population with high parental income and a college educated background (38). Geneva has a very culturally diverse population and the costs of the ESDM intervention program are almost completely covered (39). As a result, our sample is fairly representative of Geneva's residents socio-economic characteristics thus providing results with a very high degree of cultural and socio-economic generalizability compared to most studies in the field (see **Table 1**).

Ultimately, enrollment also depended on place availability at time of referral. The parents of all participants gave their written informed consent to the research protocol that was approved by the institutional review board of the University of Geneva. All participants were assessed in the context of the ongoing longitudinal Geneva Autism Cohort study. Twenty-two children from this same sample were already included in a previous study measuring outcome after 1 year of ESDM intervention (40). Baseline evaluations were completed at the start of the intervention and comprised behavioral measures that are detailed below. Parents also filled out questionnaires regarding medical history, as well as demographic information detailed below. Children were then assessed at 3 other time points at 6, 12, and 24 months of therapy, for a total of 4 assessments. Post-intervention data about subsequent school placement and support needs were collected. Children went onto either regular educational classrooms with varied levels of in-class paraprofessional support or special education classrooms.

Intervention

The 55 participants were enrolled in one of the 4 units of the *Centre d'Intervention Précoce en Autisme* (CIPA) in Geneva, Switzerland [Fondation Pôle Autisme (http://www.pole-autisme.ch) & Office Médico-Pédagogique], where they received 20 h a week of daily, individual intervention sessions using the Early Start Denver Model (ESDM). The ESDM is a comprehensive, evidence-based early intervention approach that promotes child learning through naturalistic developmental, and behavioral techniques (7, 41). Parents of the participants were provided with 12 h of once-a-week parent coaching sessions in the use of the ESDM model at the start of their child's program, and continued parent support sessions as needed throughout the 2-year period. The children were evaluated every 3 months using the Early Start Denver Model Curriculum Checklist for Young Children with Autism (ESDM-CC) to establish targeted and measurable learning objectives. The intervention services were provided by graduate-level therapists (at least Master's degree), who were trained within the CIPA program in the use of the ESDM approach, meeting ESDM fidelity on the ESDM Fidelity Rating System (41). Today, the team consists of 20 credentialed ESDM therapists, and the program is overseen by an ESDM certified trainer. Importantly, university background, ESDM training,

TABLE 1 | Sample characteristics over the 2 years of ESDM intervention.

Measure	At Baseline	+6 months	+12 months	+24 months	Pval (R.M. ANOVA)	Partial eta squared	0–24 mo	0–12 mo	12–24 mo
Clinical description									
ADOS CSS total [Mean (SD)]	**8.0 (1.9)**		**6.6 (2.0)**	**6.6 (2.7)**	**<0.001*** **	0.169	**0.002****	**<0.001*** **	1.000
ADOS CSS SA	**7.6 (2.0)**		**5.3 (1.7)**	**5.5 (2.5)**	**<0.001*** (G)**	0.304	**<0.001*** **	**<0.001*** **	1.000
ADOS CSS RRB	**8.0 (2.0)**		**9.1 (1.3)**	**8.4 (2.7)**	**<0.007** (G)**	0.094	0.975	**<0.001*** **	0.122
ADI-R subdomains [Mean (SD)] (*n* = 51)									
ADI-R social interactions	14.2 (5.2)								
ADI-R RRB	4.0 (2.2)								
VABS-II adaptive behavior composite [Mean (SD)] (*n* = 48)	**79.9 (9.4)**	*81.3 (12.0)*	**80.7 (12.3)**	**83.3 (16.1)**	**0.046* (G)**	0.073	0.152	1.000	**0.044***
VABS-II socialization	80.6 (9.9)	*81.0 (9.9)*	80.7 (10.2)	80.4 (12.6)	0.967 (G)				
VABS-II communication	**75.6 (12.3)**	*80.5 (14.8)*	**84.0 (17.7)**	**87.6 (19.0)**	**<0.001*** (G)**	0.377	**<0.001*** **	**<0.001*** **	**0.008****
VABS-II daily living skills	83.57 (12.4)	*85.0 (11.6)*	84.8 (11.8)	84.5 (16.8)	0.726 (G)				
VABS-II motor skills	89.5 (11.6)	*89.4 (11.0)*	89.9 (10.6)	90.1 (15.2)	0.920 (G)				
Composite DQ [Mean (SD)]	**60.1 (17.6)**	*71.7 (20.9)*	**77.0 (25.0)**	**80.0 (28.1)**	**<0.001*** **	0.325	**<0.001*** **	**<0.001*** **	1.000
Fine motricity DQ	**74.3 (17.0)**	*76.1 (16.0)*	**76.1 (18.4)**	**81.7 (25.5)**	**0.035 (G)***	0.063	0.088	1.000	0.132
Visual reception DQ	**74.6 (22.9)**	*85.3 (23.5)*	**88.6 (30.8)**	**88.6 (30.7)**	**<0.001*** (G)**	0.150	**0.003**	**<0.001*** **	1.000
Expressive language DQ	**44.0 (19.2)**	*56.8 (25.3)*	**66.4 (29.3)**	**68.7 (28.0)**	**<0.001*** (G)**	0.447	**<0.001*** **	**<0.001*** **	0.782
Receptive language DQ	**47.4 (26.2)**	*68.8 (29.0)*	**76.8 (30.1)**	**79.0 (33.6)**	**<0.001*** (G)**	0.432	**<0.001*** **	**<0.001*** **	1.000
Demographics									
Chronological age [months Mean (SD)]	28.7 (5.1)								
Gender [females Number (percentage)]	7 (12.7%)								
Parental Education [Number (percentage)] (*n* = 46)									
Elementary school or high school	21 (38.2%)								
University or Ph.D.	33 (60.0%)								
Household income [Number (percentage)] (*n* = 45)									
<60 k	14 (25.5%)								
60–140 k	18 (32.7%)								
>140 k	21 (38.2%)								

*Scores at 6 months (in italic) are indicative and were not used in the repeated measure (R.M.) ANOVA. Scores with a significant difference are highlighted in bold; *p < 0.05, **p < 0.01, ***p < 0.001. G, Greenhouse-Geisser correction applied; ESDM, Early Start Denver Model.*

fidelity rating assessment and supervision by certified trainer does not differ across the four units. The separation in four units is essentially administrative and therapists are all part of the same team sharing the same supervisors, applying identical practice.

Measures

The ADOS (which refers to the ADOS-G and its later version, the ADOS-2), is a standardized assessment which comprises a series of semi-structured social presses aimed to elicit and measure ASD symptoms (33, 34). The schedule comprises 5 different modules, adapted to the person's age and level of language. The calibrated severity score (CSS) was used to compare the total severity score as well as the restricted and repetitive behaviors (RRB) and social affect (SA) symptoms severity scores (42, 43). The ADOS were administered by a trained examiner and filmed. The members of the team who rated the video recordings were not implicated in the delivery of the ESDM intervention.

The Mullen Scales of Early Learning (MSEL) is a standardized assessment for children aged from birth to 68 months (44). It measures the child's development in five developmental domains: expressive language (EL), receptive language (RL), visual reception (VR), fine motor (FM), as well as gross motor skills (GM).

The Psychoeducational Profile—third edition (PEP-3) is a standardized assessment tool that evaluates cognitive, motor, and adaptive domains in children 2–7 years of age (45). These domains include EL, RL, FM and cognitive verbal and preverbal (CVP). The PEP-3 as well as the MSEL were administered by psychologists following the standard instructions of both evaluations.

Developmental quotient scores (DQ) were computed for each subdomain of the MSEL by dividing the individual developmental age by the chronological age and multiplying by 100 as described in 2006 by Lord et al. (46). The composite DQ was computed by calculating the average of all four subdomains' developmental ages, then dividing by the chronological age and multiplying by 100. Similarly, DQ scores were computed for the subdomains of the PEP-3 that assess domains equivalent to those of the MSEL, namely EL, RL, CVP, and FM. The PEP-3 composite DQ was derived using the same formula as described for the MSEL, and has already been used for the PEP-3 subdomains in previous studies (40). For our analyses of cognitive skills, we used the MSEL Early Learning Composite DQ. Since the MSEL was not administered for some participants ($n = 7$ at baseline, $n = 7$ after 6 months of therapy, $n = 3$ after 12 months of therapy, $n = 2$ after 24 months of therapy) we replaced the missing DQ scores by their equivalent DQ scores derived from the PEP-3. It is important to keep in mind that DQ is normalized for the age at the time of evaluation. Hence, a DQ that remains stable over time does not reflect stagnation but rather continued developmental progress. Also, a loss of DQ over time does not necessarily imply regression (a loss of skills) but rather slower skill acquisition, leading to a widening of the gap between the child's current abilities and what would be expected in typical development.

The Vineland Adaptive Behavior Scales-−2nd edition (VABS-II) is a semi-structured interview administered by a trained clinician that assesses a person's adaptive behavior (47). The domains assessed comprise communication, socialization, daily living skills (DLS) and motor skills. An overall adaptive behavior composite score (ABC) of all these 4 domains is computed.

The ADOS, VABS-II, PEP-3 and MSEL were administered at baseline, after 12 months and after 24 months of therapy. Assessment at 6 months only comprised the VABS-II and the MSEL.

We measured participant socio-economic using the total household yearly income and the highest level of education achieved by parents at baseline. The household income was divided into three subgroups that are detailed in **Table 1**. Parental educational level was first coded using the seven categories of the four-factor index of social status developed by Hollingshead (48). We then divided these categories into two groups: (1) elementary school or high school completed, and (2) college and/or graduate degree completed.

Rate of Change

For all behavioral measures acquired longitudinally (ADOS, VABS-II and DQ), we computed an individual rate of change using the following Symmetrized Percent Change (SPC) formula:

$$SPC\ \left[\%/year\right] = 100 \times \frac{(B_y - B_x)/[(B_x + B_y)/2]}{(age_y - age_x)}$$

Where B_x and B_y represent the behavioral measure acquired when the participant was aged of age_x and age_y, respectively. In other words, SPC is the behavioral difference between two timepoints relatively to the mean of the scores across these two timepoints, then divided by the time interval (in years). This results in a yearly rate of change that can be expressed as a percentage when multiplied by 100. The main advantages of using symmetrized measures of change over absolute differences (such as $B_y - B_x$) or non-symmetrized percentages [such as $(B_y - B_x) / B_x$] comprise increased statistical robustness, higher reliability in small samples, limited sensitivity to outliers, and equivalent consideration of both B_x and B_y measures (49). Also, SPC was chosen over absolute difference because it is scaled for the global developmental level of the child. Analyzing the cognitive changes using absolute differences leads to considering a gain of 10 DQ points in a child with an initial DQ of 90 as equivalent to a gain of 10 points in a child with a 60 composite DQ at baseline. In contrast, using SPC would give more weight to the gains made by the child with the lower DQ at start despite the fact that the absolute change is identical. In children with ASD and low DQ, small absolute gains have a larger impact in their adaptive behavior compared to their peers with higher DQ (50). Hence, measuring rates of change relatively to each participant global developmental level as SPC appears more clinically meaningful.

Statistical Analyses

IBM®SPSS® Statistics v26.0.0.0 for macOs (Armonk, NY: IBM Corp.) was used for all analyses. Statistical significance threshold was set at alpha = 0.05. Graphs were obtained with Prism® v8.3.0 (GraphPad Software, La Jolla California USA, www.graphpad.com) and Matlab R2018b for MacOs (MathWorks).

To test for an effect of time, a repeated measure ANOVA was performed on the whole sample for each longitudinal behavioral

measure using the scores collected at baseline, 12 and 24 months after the start of the intervention services. Greenhouse-Geisser correction was applied whenever the assumption of sphericity was violated according to Mauchly test.

Until now, methodological strategies to identify intervention-specific predictors of EI outcome include whole-sample correlations between baseline and outcome measures (29, 51), comparison between subgroups defined based on an arbitrary cut-off such as rapid vs. slow learners (52) or best vs. non-best outcome (53, 54). A promising alternative relies on the identification of distinct phenotypic subgroups within ASD (32). Defining more homogeneous subgroups based on behavioral characteristics in a data-driven manner can be achieved by applying cluster analyses (CA), a strategy that has already been used in ASD preschool studies [for a review see (32)]. To date, CA has only been applied once on children with ASD participating in an EI program (Applied Behavioral Analysis: ABA) with a special focus on language development (55). We here performed a cluster analysis (CA) using cognition (assessed with the composite DQ measure) as our main outcome measure. There are several reasons why we chose DQ over other parameters. First, it is generally the main outcome measure reported in early intervention studies, and it displays the most variability (2, 56, 57). Second, cognition has been shown to be the domain that improves the most after early intervention (58). Third, studies investigating possible ASD subtypes within ASD have shown that the most salient group differences emerge when categorized by cognitive skills (59). We used a *k*-means clustering approach to identify subgroups in terms of DQ trajectories with a maximal number of iterations set to 10 (60). We chose two variables that capture individual DQ trajectories: the composite DQ at baseline and the composite DQ SPC over the 2-year intervention period. To objectively determine the number of clusters *k* we used a two-step clustering approach as suggested by Kodinariya and Makwana (61). We used the two-step clustering algorithm developed by Chiu et al. (62) as it is implemented in IBM®SPSS® Statistics. Briefly, this method firstly divides the sample into a set of sub-clusters through a sequential approach and secondly merges the sub-clusters through a hierarchical technique based on the log-likelihood distance between them. Finally, the Akaike's information criterion is used to objectively determine the optimal number of clusters.

The cluster analysis (CA) yielded 3 optimal clusters based on the baseline composite DQ and the composite DQ SPC over 2 years (**Figure 1**) with silhouette measure of cohesion and separation equal to 0.6. The ANOVA revealed that one of these clusters exhibited significantly greater composite DQ at baseline compared to the others and was therefore named "higher cognitive at baseline" (HC, $n = 20$). Its average DQ at baseline was 78.6 ± 10.9 with a range between 64.4 and 107.9 with a SPC of 9.9 ± 5.8 %/yr. This corresponds to an average 18.3 gain for a final DQ of 96.9 ± 14.3 with a range between 64.2 and 124.3. The second "optimal responders" cluster (OptR, n = 24) was characterized by high rates of progress within the 2-year program. DQ at baseline was 51.5 ± 10.7 with a range between 21.9 and 66.8, and its average SPC was 23.8 ± 7.9%/year. This corresponds to an average 34.6 gain for a final DQ of 86.2 ± 20.8 with a range between 32.5 and 130.3. The third "minimal responders" cluster (MinR, $n = 11$) was characterized by decreased rates of progress compared to the two other clusters with an average SPC of −11.5 ± 12.0%/yr. Its composite DQ at baseline was 44.9 ± 8.1 with a range between 31.7 and 59.1. The average loss was 9.1 for a final DQ of 35.8 ± 8.9 with a range between 24.5 and 58.0. The OptR and MinR subgroups did not differ in composite DQ at baseline, with an average of 51.5 and 44.9 respectively. Together, they form a group of children with lower cognitive scores (LC) at baseline. Cluster differences over composite DQ at baseline and composite DQ SPC are illustrated on **Figure 1**. Detailed analyses are reported in **Supplementary Tables 1, 2**.

Demographic, socio-economic measures and behavioral measures at baseline were compared between clusters using one-way analysis of variance (ANOVA) or chi-square test. We used a Bonferroni correction for multiple testing on the subdomains of the same clinical evaluation (e.g., the subdomains of the VABS-II), setting the statistical significance at 0.05/number of subdomains. When an ANOVA reached statistical significance, *post-hoc* comparisons between clusters were performed using multiple *T*-tests with Bonferroni correction and statistical significance set at 0.05/number of clusters.

We then applied the same strategy to compare the SPC between clusters. We performed analyses on the following SPC: from baseline to 6 months, from baseline to 12 months, from baseline to 24 months of therapy.

Finally, we focused on the two LC clusters which showed no differences in their composite DQ at baseline to explore whether any other behavioral measure could help classifying them. To do so, we used logistic regression models. More specifically, we selected all behavioral measures that differed between OptR and MinR on *post-hoc T*-tests at baseline. Then, we performed a multivariate logistic regression using the selected measures. Whenever a composite score and one or more subdomain scores of the same test were selected, we preferred the composite measure to minimize potential collinearity between variables in the model. Then, we used the same strategy for the SPC measures during the 6 first months of intervention, and ultimately with those of the 12 first months.

Sample Size

Once the three clusters solution obtained, we were able to compute the estimated power to detect differences between groups. Based on a sample of 55 children divided in three clusters and assuming an alpha of 0.05 using ANOVA, we calculated 80% power to detect group differences of at least 0.430 effect size.

RESULTS

Whole Sample Trajectories

Descriptive measures collected at each visit are reported in **Table 1** for the total sample. The children were aged from 15.3 to 42.0 months at the beginning of the intervention (average: 28.7 ± 5.1 months). The average composite DQ of the entire group at baseline was 60.1 ± 17.6 (range: 21.9–107.9). As a group, all 55 children receiving ESDM showed a significant

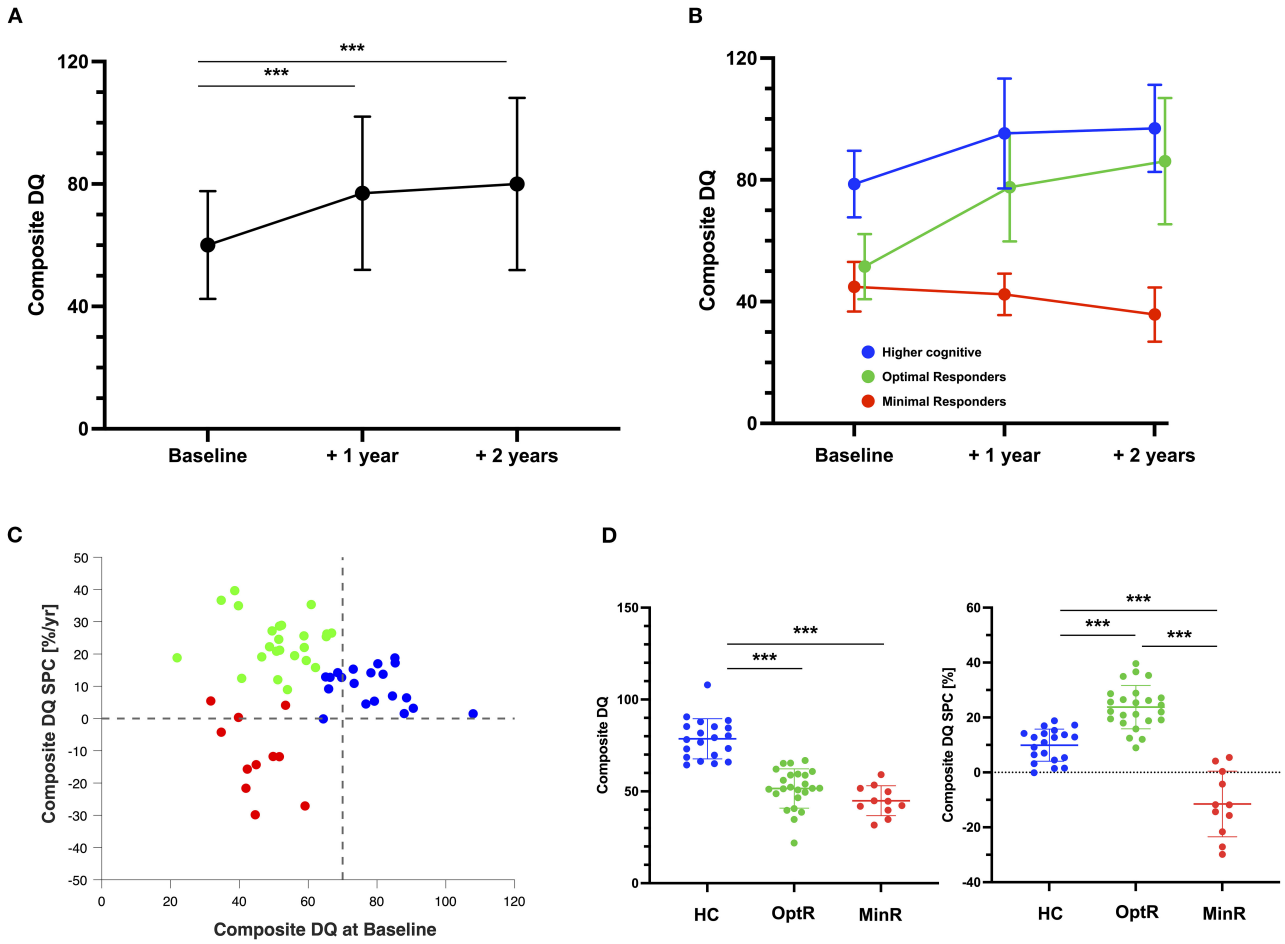

FIGURE 1 | (A) Composite DQ trajectory of the total sample over the 2 years of intervention. Significant results of repeated measure ANOVA are displayed. **(B)** Composite DQ trajectories of the three subgroups parsed by the cluster analysis. **(C)** Individual values of the two measures used in the clustering analysis algorithm (composite DQ at baseline and DQ SPC over the 2 years of interventions). Color code represents the cluster membership of each participant after the application of the cluster analysis. **(D)** Differences between the three subgroups on the two measures that were used to parse them. ***$p < 0.001$. DQ, Developmental quotient; HC, Higher cognitive; MinR, Minimal responders; OptR, Optimal responders; SPC, Symmetrized percent change.

decrease in their total level of symptom severity (ADOS CSS) (see **Table 1**). This improvement was driven by a decrease in the Social Affect (SA) domain. On the contrary, the RRB symptom severity increased over time. We found that these changes occurred mainly during the first year of intervention and that CSS (both RRB and SA) were stable during the second year of intervention. In parallel, participants' developmental scores improved. This improvement was significant in all subdomains (i.e., FM, VR, RL and EL). As for the measures of symptom severity, all changes in cognition were significant during the first year of therapy but not the second one, except for FM rates of change during first year that did not reach significance level in *post-hoc* analyses. Finally, increase in DQ was accompanied by an improvement of adaptive functioning as measured by the VABS ABC. This increase was significant during the second year of intervention only. More precisely, participants made significant gains in the communication subdomain which occurred both during the first and the second year of intervention. All statistically significant results are detailed in **Table 1**. Concerning the type of schooling after the intervention, 35 participants (63.6%) joined a public regular education classroom with individual educational support. One participant (1.8%) joined a regular education classroom without any support. Four children (7.3%) entered a private school that provided in-class support in a regular education classroom. Finally, 15 participants (27.3%) entered special education program within the public-school system.

Parsing the Heterogeneity in Treatment Response

Difference Between the Three Subgroups at Baseline

We found no differences between clusters for parental educational attainment or household income (see **Supplementary Table 1**). Inclusion procedure resulted in a sample that was relatively homogeneous in age at baseline (28.7 ± 5.1 months). Yet we compared age at baseline between groups to exclude this variable as a confound factor and found

no difference regarding age at baseline. When looking at DQ at baseline, we found that HC showed higher scores in all DQ subdomains compared to both other clusters. Considering adaptive behavior, HC exhibited higher scores in ABC as well as in the communication subdomain compared to both other clusters. HC also showed a higher score in adaptive socialization and motor skills compared to MinR. All statistically significant results of analyses on the DQ and the VABS-II across the three subgroups at baseline are illustrated on **Figure 2**. There was no difference in the total ADOS CSS. In the ADOS subdomains, we found that HC exhibited lower RRB compared to MinR. Besides, the only difference between MinR and OptR was in global adaptive functioning (VABS ABC), MinR showing lower scores (70.7 ± 5.2) at baseline compared to OptR (78.5 ± 7.5).

Differences Between Subgroups in Rates of Change Over 6, 12, and 24 Months of Intervention

We found that over the 2 years of therapy, OptR exhibited higher rates of change compared to the other two subgroups in cognition (composite DQ as well as VR, FM and RL subdomains). They also showed higher rates of change in adaptive behavior compared to MinR (VABS-II ABC, in socialization, communication, and DLS subdomains) (see **Supplementary Table 2**). These differences between MinR and OptR were already present within the 12 first months of therapy in the communication subdomain. Also, we found that MinR exhibited slower rates of change during the total time of intervention compared to both other subgroups in cognition (composite DQ, VR, FM and EL) as well as in adaptive behavior (VABS-II ABC, socialization, communication and DLS). Differences in the rates of change of composite DQ, VR and EL between MinR and OptR were already significant during the first year of intervention.

Finally, we found that OptR already exhibited faster rates of change in composite DQ, and adaptive functioning (VABS ABC) compared to MinR (**Figure 3**) after only 6 months of intervention. These differences in early DQ and VABS ABC rates of change were driven by RL and adaptive communication SPC.

We did not find any difference in the rates of change of symptom severity (total ADOS, SA and RRB) between the three subgroups during the time of intervention.

Subgroup Classification Based on Early Rates of Change

Minimal responders (MinR) and optimal responders (OptR) showed no difference on the composite DQ at baseline and were both considered to have lower cognitive scores at baseline (LC). They were thus selected for our classification analyses to address the potential of clinical measures at baseline as well as their early rates of progress to classify them. At baseline, these two subgroups differed in VABS ABC. Logistic regression based on this parameter allowed an overall classification precision of 70.4% (5 out of the 11 MinR and 21 out of the 23 OptR were classified

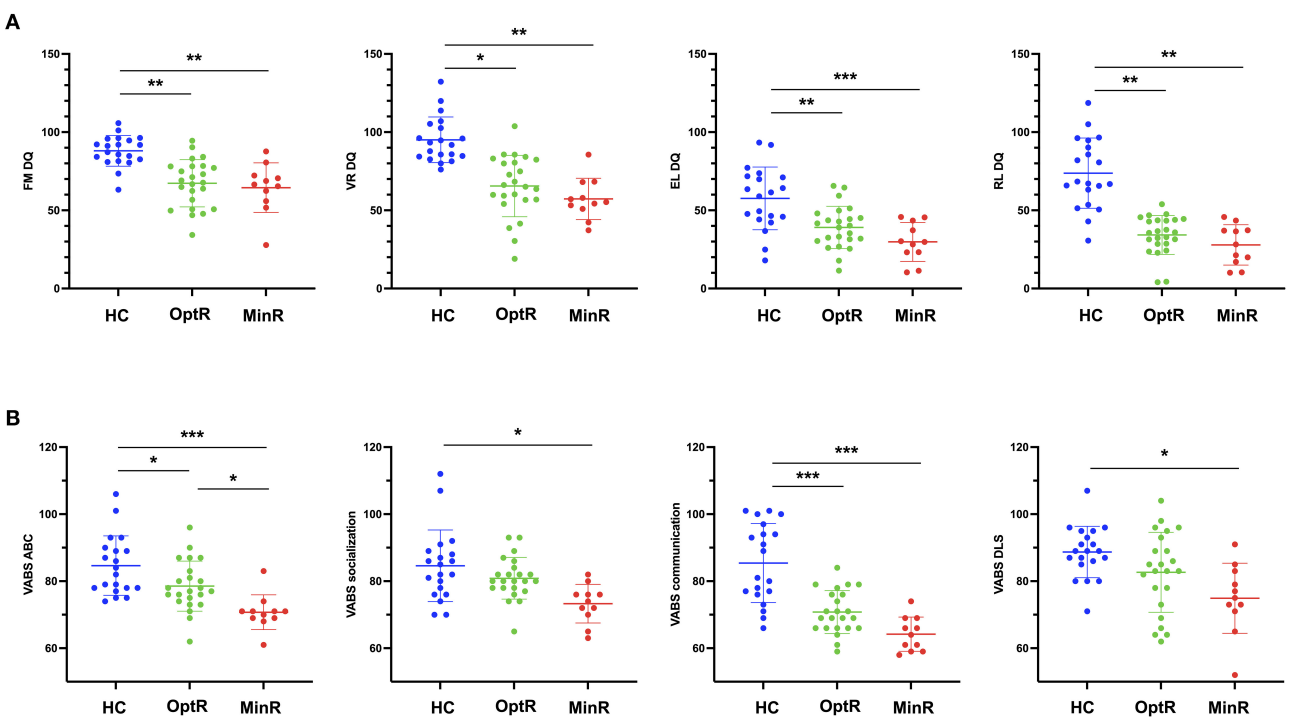

FIGURE 2 | (A) Statistically significant differences in DQ subdomains between subgroups at baseline. **(B)** Statistically significant differences in VABS-II ABC and VABS-II subdomains between subgroups at baseline. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. ABC, Adaptive behavior composite; DQ, Developmental quotient; EL, Expressive language; FM, Fine motor; HC, Higher cognitive; MinR, Minimal responders; OptR, Optimal responders; RL, Receptive language; VABS-II, Vineland adaptive behavior scale; VR, Visual reception.

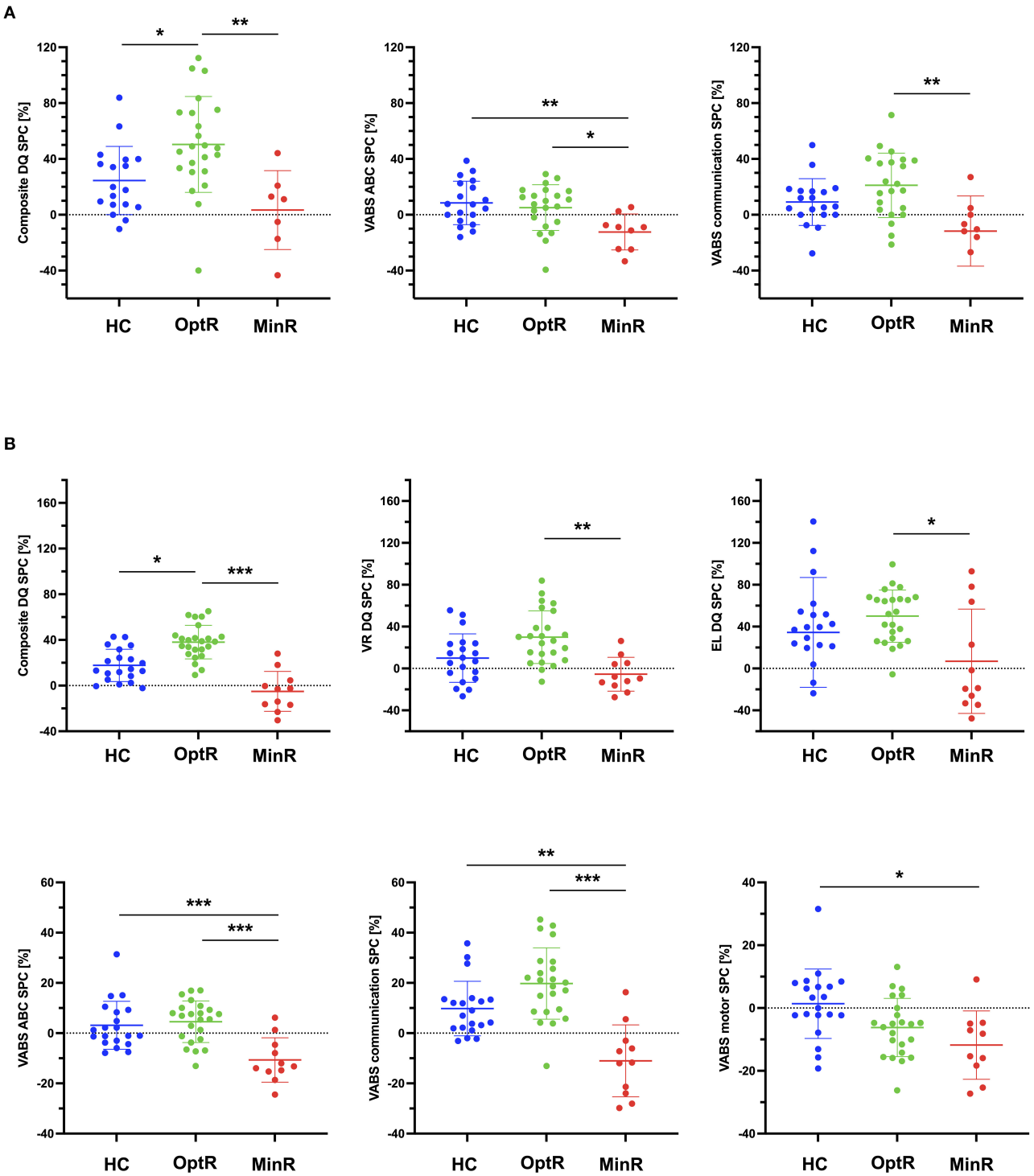

FIGURE 3 | (A) Statistically significant differences between subgroups in the rates of change of behavioral measures (DQ and VABS-II) within the first 6 months of intervention. **(B)** Statistically significant differences between subgroups in the rates of change of behavioral measures (DQ and VABS-II) within the first 12 months of intervention. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. ABC, Adaptive behavior composite; DLS, Daily living skills; DQ, Developmental quotient; EL, Expressive language; FM, Fine motor; HC, Higher cognitive; MinR, Minimal responders; OptR, Optimal responders; RL, Receptive language; SPC, Symmetrized percent change; VABS-II, Vineland adaptive behavior scale; VR, Visual reception.

correctly). The model was significant ($\chi^2 = 10.0$, $p = 0.002$) and explained 35.5% of the variance (Nagelkerke R^2).

Within the first 6 months of therapy, MinR showed slower SPC in the VABS-II ABC and in the composite DQ. Logistic regression based on these two variables allowed a partition of MinR and OptR with a 85.2% overall correct classification rate. Nineteen out of the 21 OptR included in the model and 4 out of the 6 MinR included were classified correctly. The logistic regression model was statistically significant ($\chi^2 = 10.2$, $p = 0.006$) and explained 48.0% of the variance (Nagelkerke R^2). The prediction equation was the following: $0 = -0.040 * DQ\ SPC - 0.112\ VABS\text{-}II\ ABC\ SPC - 0.92$.

Within the first 12 months of therapy, OptR exhibited higher SPC in both the VABS-II ABC and the composite DQ. Logistic regression performed with both measures reached a 94.3% rate of overall correct classification between OptR and MinR. Twenty-two out of the 23 OptR included in the model and 9 out of the 11 MinR included were classified correctly. The logistic regression model was statistically significant ($\chi^2 = 22.5$, $p < 0.001$) and explained 67.5% of the variance (Nagelkerke R^2). The prediction equation was the following: $0 = -0.070 * DQ\ SPC - 0.167\ VABS\text{-}II\ ABC\ SPC - 0.020$.

Ultimately, we combined the information at baseline with the rates of change to see if the classification model was enhanced (**Figure 4**). Combining VABS ABC at baseline with VABS ABC and DQ SPC within the first 6 months we achieved a model with 96.3% overall precision. Five of the 6 MinR and all of the 21 OptR were classified correctly (Nagelkerke $R^2 = 70.2\%$; $\chi^2 = 16.6$, $p = 0.001$; $0 = -0.268 * VABS\text{-}II\ ABC - 0.049 * DQ\ SPC - 0.151 * VABS\text{-}II\ ABC\ SPC + 19.450$). Combining VABS ABC at baseline with VABS ABC and DQ SPC within 12 months the model reached 94.1% overall precision. Ten of the 11 MinR and 22 of the 23 OptR were classified correctly (Nagelkerke $R^2 = 75.5\%$; $\chi^2 = 26.4$, $p < 0.001$; $0 = -0.188 * VABS\text{-}II\ ABC - 0.040 * DQ\ SPC - 0.191 * VABS\text{-}II\ ABC\ SPC + 13.107$).

In other words, it would have already been possible for a clinician to classify a child as being an OptR or MinR with 96.3% of accuracy after 6 months of intervention based on the child's adaptive functioning at baseline and its rates of change in adaptive functioning and cognition.

DISCUSSION

In the present study, we analyzed data from one of the largest samples of children who underwent 2 years of intensive (20 h per week) and individualized ESDM intervention to identify predictors of their developmental outcome. Overall, we observed that preschoolers in our sample made significant cognitive progress and adaptive skill gains over the 2 years of intervention (see **Figure 1A**). Improvements in the current sample allowed 72.7% of the children to enter a regular education classroom post-intervention, which in Geneva requires the child to have near peer-level functioning. These results are consistent with those reported in other studies on ESDM-based intervention (2, 63). More specifically, our sample exhibited an average change in DQ (+19.9 points) that is very close to the one described in the randomized controlled trial (RCT) study by Dawson et al. (2), which reported 18 points of cognitive gain, an average significantly greater than that of their control group. In parallel, a naturalistic study that explored developmental trajectories in preschoolers with ASD who were not enrolled in any specific therapeutic program reported an average DQ gain of only 6.3 points between 24 and 48 months of age (64). The average initial DQ in the cited study (63.6 ± 11.5) was similar to ours (60.3 ± 18.0). Considering similarities in the outcome between our results and previous ESDM studies as well as differences with naturalistic studies, one can infer that ESDM intervention in our study had a causal effect on the improvements observed at the whole group level. These results therefore highlight the possibility of implementing ESDM in Europe as effectively as

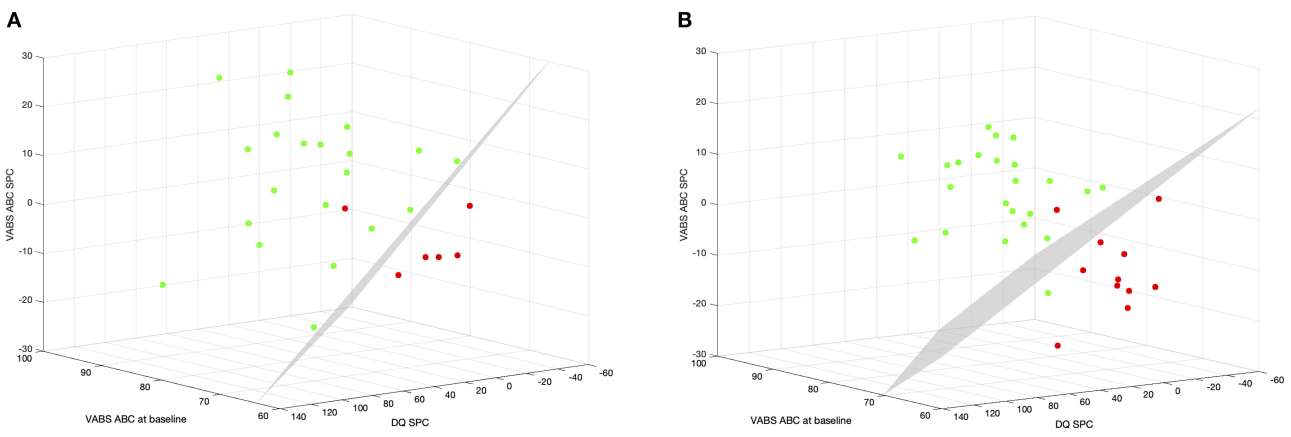

FIGURE 4 | (A) Multinomial logistic regression between MinR and OptR using their rates of change within the 6 first months of intervention in Composite DQ and VABS-II ABC, as well as their VABS-II ABC at baseline. Color code corresponds to subgroup membership (red: MinR, green: OptR). Decision boundary is represented in a dotted line. **(B)** Multinomial logistic regression between MinR and OptR using their rates of change within the 12 first months of intervention in Composite DQ and VABS ABC, as well as their VABS-II ABC at baseline. ABC, Adaptive behavior composite; DQ, developmental quotient; SPC, Symmetrized percent change; VABS-II, Vineland adaptive behavior scale.

in the US, despite differences in culture and health care system. They also support the cost-effectiveness of ESDM intervention, with improvements in cognition and adaptive behavior known to reduce subsequent school-based support needs, offsetting costs associated with early intensive intervention (17–19).

This study also aimed to determine whether preschoolers with ASD who participated in a 2-year NDBI intervention program (here ESDM in an individual setting, or I-ESDM) could be separated into distinct subgroups based on their cognitive trajectories over time. To achieve this, we used a *k*-means cluster analysis (CA) approach with cognitive abilities at baseline and cognitive rates of change over time as variables. CA yielded three groups: 36.4% ($n = 20$) of children with a mild cognitive delay at baseline that displayed a globally good outcome (Higher cognitive at baseline: HC), and two groups of children constituting the lower cognitive scores at baseline group (LC) that had very different outcomes. The first group of LC, which represented 43.6% ($n = 24$) of the entire sample, underwent significant cognitive and adaptive skill improvements (Optimal responders: OptR) while the second group of LC, which represented 20.0% ($n = 11$) of our sample, showed slower overall progress, and saw a widening of the developmental gap over time in cognition and adaptive behavior compared to same aged peers (Minimal responders: MinR). The clear distinction between toddlers with mild cognitive delay (HC) and those with the more severe cognitive delay at baseline (LC) observed in our sample is also reported in previous studies that applied CA to preschoolers with ASD (65–67). These studies identified at least two subgroups categorized as "high" and "low-functioning" based on early cross-sectional cognitive measures. One of the main differences in the present study is that we included a longitudinal variable in our CA (i.e., the rate of cognitive change) and were therefore able to further define our subgroups of LC children based on individual cognitive trajectories over time that a cross-sectional CA would have failed to capture. In our second analysis, we aimed to uncover potential predictors of outcome by evaluating how we can predict a child's cluster membership. Amongst the LC subgroups (MinR and OptR), we found one difference at baseline in general adaptive functioning (see **Supplementary Table 1**). More importantly, we noted a significant difference between their rates of change in cognition and adaptive skills within the first 6 months of intervention. These differences were mostly driven by the progress in receptive language and adaptive communication. Using a logistic regression model, we showed that these early rates of change combined to differences at baseline predicted at 96.3% attrition to either the MinR or OptR subgroups. This means that higher adaptive functioning skills at baseline combined to early, rapid developmental progress by 6 months of intervention allowed an accurate classification of subsequent developmental pattern.

Our analyses of the HC subgroup suggest that a mild cognitive delay (78.6 ± 10.9 of composite DQ) at the start of an ESDM intervention is associated with an alleviation of the delay in cognitive skills (+9.9% DQ per year) and adaptive behavior (+4.2% ABC per year) over the course of treatment. In addition, children in the HC group exhibited higher levels of adaptive skills compared to other subgroups (84.9 ± 9.2 of ABC) at baseline, especially in the VABS-II domain of communication (84.7 ± 8.9). All HC children except for one were able to continue into a regular education classroom following the intervention. With a DQ of 64 at both the beginning and the end of the intervention, this child was the only one in the HC group with a DQ value lower than 80 at the end of the intervention. Overall, our HC subgroup results suggest a positive outcome (in terms of cognition, adaptive functioning and schooling) in preschoolers with a mild delay in cognition and communication at baseline after receiving an individualized and intensive ESDM intervention. A recent review concluded that a higher cognitive level at baseline is a good predictor of positive outcome after various types of EI (68). Also, previous studies focusing on another type of intervention (Applied Behavioral Analysis: ABA) reported that higher abilities in adaptive behavior (52, 69) as well as in language (70) constitute predictors of good outcome. This might suggest that mild delays in cognition and communication could represent a common predictor of good outcome among various EI approaches. These findings will need to be further explored with future RCT that assess the specific causality of ESDM intervention within these results. A practical implication of our findings concerning the HC subgroup is that clinicians who refer a toddler with a mild developmental delay at baseline to an ESDM program can be relatively confident that there will be a good outcome in cognition and adaptive behavior by the end of the intervention.

Apart from the HC group, the rest of the sample included children presenting a severe cognitive delay at baseline (Lower Cognitive, or LC). These children presented drastically different cognitive trajectories of change over time and were attributed to two distinct subgroups: OptR and MinR. Despite their severe cognitive delay at baseline (average DQ of 51.5 ± 10.7), the 24 children that composed the OptR subgroup greatly improved their cognitive and adaptive skills over time and 79.2% of them were able to join a regular education classroom with in-class support. On the other hand, the 11 children in the MinR subgroup had a similar level of cognitive impairment at baseline (average DQ of 44.9 ± 8.1), however their cognitive and adaptive functioning scores did not improve over time. Furthermore, the developmental gap between continued to widen, despite receiving intensive early intervention. Only 2 out of 11 (18.2%) MinR children joined a regular education classroom following the intervention. One clinical implication of our analyses of OptR and MinR at baseline is that the OptR constituted most of the LC children (68.6%) thus supporting the *a priori* that most toddlers who present with lower cognitive scores at intake display a positive outcome after receiving an individual intensive ESDM intervention. Nonetheless, a better understanding of the factors (behavioral, biological, and environmental) that are associated with MinR remains necessary to develop more targeted clinical recommendations. For instance, future studies including more participants with comorbid conditions such as epilepsy should investigate whether they represent moderator of outcome. Furthermore, they could determine whether the additive effect of various genetic mutations may moderate the outcome (71). In our sample, four participants had a reported genetic finding with a potential causal effect in ASD. Two of them were in the MinR group one in OptR and one in HC. Yet, the

sample is far too small to draw any conclusion on the matter and future studies should address how genetic alterations modulate the RTT. The observation of two distinct trajectories of change in children with larger cognitive impairments at baseline could shed new light on the inconsistencies that exist between various studies that measured cognitive response to EI within LC preschoolers with ASD. For instance, one previous study concluded that children with this kind of profile only improve in fine motor skills and receptive language but not in adaptive behavior after receiving an early and intensive ABA intervention (72). Other studies focusing on ESDM reported an association between lower cognitive level at baseline and high cognitive gains (30, 31). One can hypothesize that the inter-individual heterogeneity of outcome reported by previous studies (30, 31, 72), as well as the differences in their results were potentially due to the existence of two latent subgroups (MinR and OptR) that may have driven results in opposite directions. Our results thus advocate for a more systematic subgroup phenotyping, including longitudinal variables, in future studies focusing on the clinical outcome of EI to better describe the phenotypic heterogeneity within LC preschoolers with ASD.

Finally, our results suggest that the outcome after 2 years of intervention for children with LC at baseline can be predicted by the end of the six first months of intervention with high accuracy. Adaptive functioning was the only clinical parameter that could help distinguish OptR from MinR at baseline, allowing an overall classification precision of 70.4%. Nonetheless, based on this single variable only 45.5% of MinR could be classified correctly resulting in a relatively poor sensitivity in MinR identification at baseline. Sensitivity to OptR was largely higher with 91.3% of them correctly classified at baseline. Thus, the clinical interest for using the VABS-II alone at baseline to discrimate between LC (OptR and MinR) appears very limited. Nonetheless, the OptR group's rates of change appear to be significantly higher than those observed in the MinR group within the first year for cognitive and adaptive skills (especially in communication). Our results are in line with those of Sallows and Graupner (52), who reported cognitive gain during the first year of an early and intensive ABA intervention as one of the best predictor of outcome at the end of the intervention. Furthermore, we found that based on adaptive functioning at baseline combined to the rates of change in cognition and adaptive functioning within the first 6 months of intervention, we could infer the outcome after 2 years of intervention. Together, these conclusions might shed light on the timing of RTT, and when children can be considered as "*non-responders*" as raised by Vivanti et al. (13). Indeed, our analyses suggest that the first 6 months to a year of intervention offers critical information about how a child will respond to ESDM intervention over time, and leads us to question whether an early, clear response to intervention can predict an optimal response overall. The emphasis on this early response to intervention as a predictor of long-term outcome has several clinical implications. One of them would be the importance of implementing regular, standardized follow-ups to measure children's cognition and adaptive behavior in the first 6 and 12 months, in addition to the systematic ESDM *Curriculum Checklist* (ESDM-CC) that is currently used in the model. An alternative could lie in the development of a standardized way to use the ESDM-CC to track the rate of developmental change and ultimately the post-intervention outcome. This type of early standardized follow-up could potentially alert the clinician of difficulties a MinR child might face. This does not mean that MinR should be given less resources in terms of intervention. Our results show that despite a less optimal response compared to others, MinR show improvements in their raw scores. In the perspective of personalized medicine, future studies should determine what is the best intervention for these children. It might be possible that they would benefit from earlier or longer or more intensive intervention. It could also be that another type of EI (other than ESDM) would provide them a more optimal outcome. More research is needed to understand what supports or program enhancements would allow a child with a slower response to intervention to have a more optimal outcome.

In summary, our results show that despite the lack of individual reliable predictors of outcome for children with ASD who present severe cognitive delays at baseline, the consideration of their early dynamic behavioral parameters may help predict their overall response to intervention. Further RCTs that explore the trajectories of subgroups similar to ours are needed to determine the precise effect of the ESDM on children with MinR and OptR profiles. More specifically, we need to understand whether ESDM helps OptR improve their outcome or if it prevents MinR from falling even further behind developmentally, or both. Another hypothesis to be addressed is whether ESDM has an influence in the relative number of participants that are affected to each subgroup—i.e., whether some OptR participants would have been MinR if they had not undergone an ESDM based therapy. Future research on the specific effects of ESDM on each subgroup could result in improved therapeutic guidelines that are more tailored to each child's individual developmental trajectory. Our study provides relevant variables that should be explored by future research at the beginning and during the very first months of an ESDM intervention.

LIMITATIONS

Despite being one of the largest samples of preschoolers who benefited from a 2-year intensive and individualized ESDM program, the sample size of the present study limits the number as well as the size of subgroups that can be detected by a cluster analysis. Nevertheless, we took care to respect the commonly accepted prerequisites of cluster analyses, including the minimum sample size in each group or the number of factors in the analyses given the overall sample size (73, 74). It is possible that studies performed on larger samples could achieve more fine-grained subgrouping on a similar population based on the same measures and could lead to bigger subgroups, in turn increasing the statistical power to detect differences at baseline between lower cognitive clusters that we could not highlight.

Another limitation that is a direct consequence of the previous one lies in the choice of the main outcome. We chose parameters related to cognitive skills as the main clustering factors. However, it would have been possible to use other measures such as

level of ASD symptoms, adaptive skills or even a combination of these two. The inclusion of more variables in the model could help in defining a larger number of clusters and therefore increase our understanding of the heterogeneity of ASD in a refined manner. However, this was not possible in the present study, because of the limited sample size. The addition of more variables in the model and the multiplication of clusters would have violated the cluster analysis assumptions, making its interpretation invalid. Studies with larger samples should include more clinical parameters and could also use outcome variables suggested by parents (75).

Within our sample, 7 children did not have their DQ at baseline assessed with the same test as the rest of the sample. Indeed, these 7 children were tested with the PEP-3 while the others were tested using the MSEL. Although the scores obtained *via* these two assessments show a strong consistency within our sample (Cronbach $\alpha = 0.914$, $n = 44$), it is not possible to affirm that they are equivalent due to their different design. Yet, the clustering analysis applied on the sample with the 7 children excluded yielded the same cluster solution. Nonetheless, this divergence in the test used for a minority of our children should be kept in mind when interpreting the results.

A last limitation here lies in a lack of a lower cognitive (LC) who did not undergo an ESDM intervention making difficult to evaluate the causality of ESDM intervention in the observed outcome of this specific population. Nevertheless, Hedval et al. reported that 87.7% of the preschoolers with ASD and LC at baseline (<70 of DQ) still had a DQ lower than 70 when assessed after 2 years without receiving any EI (76). Moreover, their delay in adaptive functioning worsened in all the VABS-II subdomains except for communication at the group level. In contrast, in the present study LC children with similar developmental pattern (MinR) only constituted 31.4% of our LC group, while the other LC participants (OptR) exhibited large improvements in DQ as well as in adaptive behaviors. Considering these results, one can infer a causal effect of ESDM in the progress made by children with important cognitive delay at start. The specific effects of ESDM compared to other types of EI still needs to be addressed with future RCT.

CONCLUSION

In this study, we applied a cluster analysis to the largest European sample of preschoolers with ASD who participated in an ESDM program for 20 h a week over a 2-year period. Overall, we found that ASD symptom severity decreased, and cognitive delay improved over the intervention period. Furthermore, the cluster analysis suggested three main patterns of cognitive trajectories over time. First, children who displayed mild cognitive and adaptive behavioral delays at baseline tended to have a good developmental prognosis, finishing their 2 years of early intervention with cognitive and adaptive behavior scores within the normal range. Second, children who presented with severe cognitive delays at the start of their early intervention exhibited two dramatically different patterns of developmental trajectories. About a third of these children continued to fall behind developmentally, despite intensive therapy services. The two remaining thirds of the children, who presented with lower cognitive and adaptive behavior scores at the beginning of treatment, exhibited early and important gains in cognition and adaptive behavior which continued for the duration of the 2 years of intervention. We found that the two lower cognitive subgroups differed in their global adaptive functioning at baseline, although this parameter alone shows a limited sensitivity in identifying the children who will show slower gains. Nevertheless, our results suggest that it may be possible to predict, after only 6 months of early intervention, and with very high levels of accuracy, whether a child will have an overall minimal or optimal response to treatment, based on their early gains in cognition and adaptive behavior combined to their adaptive functioning at baseline. These results advocate for close monitoring using standardized cognitive and adaptive behavioral testing during the first 6 months of intervention, especially for children that exhibit a clinically significant cognitive delay at baseline. Having an understanding early-on of how a child is responding to early intervention could alert clinicians and parents to the need to adapt and enhance the child's treatment plan. Future studies are needed to replicate these findings, and to evaluate the kinds of treatment adaptations that would optimize child outcome for each ASD subgroup. Also, there is a need for longitudinal studies that provide a long-term follow-up in the years following the end of early intervention, to be able to assess whether the patterns of cognitive profiles and response to treatment observed remain stable over time. Overall, our results advocate for a more systematic use of subgroup phenotyping that includes longitudinal parameters when assessing the efficacy of an early intensive intervention, to better decipher the great heterogeneity of behavioral dynamics in treatment response.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, on a reasonable request.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Swissethics—Commission d'éthique Suisse relative à la recherche sur l'être humain. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

MS conceived and designed the study. MF, MG, NK, and FR participated in the data acquisition. MG and FR prepared and analyzed the data under the supervision of MS. MG and FR wrote the manuscript with the inputs from all other authors. All authors participated in interpretation of results and read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpsyt.2022.835580/full#supplementary-material

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BRIEF RESEARCH REPORT
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Relationship Between MR Spectroscopy-Detected Glutamatergic Neurometabolites and Changes in Social Behaviors in a Pilot Open-Label Trial of Memantine for Adults With Autism Spectrum Disorder

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Background: The neurobiology underlying ASD is largely unknown but altered neural excitability/inhibitory ratios have been reported. Memantine is an N-methyl-D-aspartate (NMDA) glutamatergic antagonist studied for the treatment of core ASD symptoms, with mixed results. We examined whether glutamatergic levels were associated with and predicted response to memantine in an exploratory pilot study.

Methods: Ten adult participants with ASD underwent proton magnetic resonance spectroscopy (^{1}H-MRS) imaging at baseline and behavioral assessments before and after 12-weeks of open-label memantine. Post-treatment scores on Clinical Global Impressions–Improvement (CGI-I) for social interaction were the primary outcome measure, and scores on the Social Responsiveness Scale (SRS) were included as a secondary outcome. LCModel was used to quantify the concentrations of Point RESolved Spectroscopy-detected glutamate+glutamine (Glx) (and other neurometabolites, i.e., N-acetylaspartate, NAA; creatine+phosphocreatine, Cr+PCr, and myo-inositol, Ins), within the left dorsolateral prefrontal cortex (LDLPFC) and right (R) posterolateral cerebellum. SPM was used to perform brain tissue segmentation within the spectroscopic voxels. CGI-I scores post-treatment were used to classify the participants into two groups, responders (scores 1–3; $n = 5$) and non-responders (scores 4–7, or withdrew due to increase behaviors; $n = 5$). Independent samples t-tests, partial correlations and linear hierarchical regression models (SPSS) were used to determine between-group differences in neurometabolite concentrations and associations between neurometabolites and behavioral scores.

Results: Responders and non-responders did not significantly differ in Glx levels in any region of interest, but differed in NAA levels in LDLPFC (higher in responders vs. non-responders). Although changes in CGI-I social scores were not correlated with Glx in any region of interest, the linear hierarchical regression did reveal that Glx and Ins levels in LDLPFC were predictors of post-treatment CGI-I social scores. Changes in SRS scores were correlated with baseline Cr+PCr levels in the LDLPFC.

Discussion: Our pilot data suggest that baseline Glx, a marker of glutamatergic neurotransmission, did not directly predict response to memantine for social outcomes in adults with ASD. However, interactions between Glx and the neurometabolite associated with glial integrity (Ins) may help predict treatment response. Further, those with highest baseline NAA, a putative neuronal marker, and Cr+pCr, a brain energy metabolism marker, were the best responders. These preliminary results may explain some of the mixed results reported in previous memantine trials in ASD. Future studies will need to examine these results in a larger sample.

Keywords: autism, glutamate, magnetic resonance spectroscopy - MRS, social outcomes, memantine

INTRODUCTION

Autism spectrum disorder (ASD) is a behaviorally defined, complex neurodevelopmental disorder characterized by early childhood onset of marked difficulties with social interaction and communication and the presentation of restrictive, repetitive patterns of behaviors and interests (1). Its phenotypic heterogeneity makes studying and treating ASD a challenging task (2). Currently, only two FDA approved drugs, risperidone and aripiprazole, are available to treat ASD and they are primarily used to manage severe irritability and aggression associated with ASD (3). Neither have shown conclusive benefit for the core features affecting social and communication skills in ASD and our understanding of the underlying neurobiology is only beginning to emerge. Some research indicates that a disrupted balance between excitation (glutamatergic) and inhibition (gamma amino-butyric acid, GABAergic) may be a primary underlying mechanism of the ASD phenotype in some individuals (4, 5). Further, postmortem studies of the ASD brain indicate potential alterations in these components of the excitation-inhibition balance in individuals with ASD (4, 6–8). For instance, a number of postmortem studies have indicated deficits in expression of glutamatergic markers, and altered minicolumnary morphometry specific to the dorsolateral prefrontal cortex of individuals with autism (9). Similarly, postmortem studies have also identified the cerebellum as a region where glutamate and GABAergic abnormalities are consistently identified in individuals with autism (6, 9). In fact, animal studies provide preliminary evidence that GABAergic abnormalities in the cerebellum are directly related to glutamate transmission and release in the prefrontal cortex in autism models indicating a possible interplay between these two important regions in the neuropathology of autism tied to the excitation-inhibition imbalance hypothesis (10).

^{1}H-MRS is a non-invasive neuroimaging tool that can be used to examine biochemical profiles of brain tissue and has identified different neurometabolic alterations associated with different psychiatric and neurological conditions, including ASD (11). Specifically, 1H MRS studies in ASD have demonstrated alterations in various cortical and subcortical regions of the brain, including the left dorsolateral prefrontal cortex (12) and cerebellum (15) which have shown abnormal levels of the 1H-MRS-detected biomarkers of glutamatergic neurotransmission, Glx (glutamate-glutamine complex) or glutamate (11, 13–16). Thus, drugs that can modulate the balance between excitation and inhibition in the brain may be beneficial for some patients with ASD and the use of 1H-MRS to determine if levels of a glutamatergic biomarker in the cerebellum or dorsolateral prefrontal cortex can predict response to treatments that target the balance between excitation and inhibition in the brain would be an innovative breakthrough toward providing precision medicine treatments for patients with ASD.

Memantine, a moderate affinity N-methyl-D-aspartate (NMDA) glutamate receptor antagonist that attenuates glutamatergic excitation, is an FDA approved treatment for Alzheimer's disease and is known to improve communication abilities in this population (17). A few small studies have shown some effectiveness of memantine in the treatment of social and communication aspects of ASD (18–20). Considering these cognitive and behavioral outcomes and the impact of memantine on excitatory-inhibitory balance, there has been significant interest in the potential use of memantine to target core symptoms in patients with ASD. However, in a recent randomized, controlled trial, memantine was not effective for targeting social withdrawal in ASD (21). Nonetheless, it is possible that differences in treatment response could be due to the heterogeneity in the nature of the excitatory-inhibitory balance between different patients with ASD, thereby causing a variation in response. Perhaps our understanding of the discrepancies between the small studies that documented evidence of beneficial effects of memantine in treating social and

communication deficits associated with ASD and the negative larger clinical trial could be clarified with better understanding of aspects of the biological heterogeneity of the disorder (18–21). In such a scenario, a biomarker to predict each individual patients' treatment response would be invaluable for increasing treatment efficacy and decreasing trial and error.

The current pilot, clinical follow-on study used ^{1}H MRS to examine whether Glx was associated with and could be used to predict treatment response to memantine in an open-label trial in adults with ASD. Since there is robust evidence supporting the role for the dorsolateral prefrontal cortex (DLPFC), the cerebellum, and their interconnection in the excitation-inhibition balance neuropathology of autism as described previously (6, 9), these two regions were selected as the regions of interest (ROI) for the current study. Specifically, our previous work had shown that the ratio between excitation (Glx) and inhibition (GABA) in the right (R) cerebellar hemisphere as well as connectivity between the left (L) DLPFC and the R cerebellum were associated with measures of social communication, (32) suggesting that these areas are reasonable target regions of interest to examine whether MRS markers of glutamatergic neurotransmission might predict response on social communication to glutamatergic antagonists. Additionally, abnormalities in the posterolateral cerebellar hemispheres appear to be associated with language and social communication, (37, 38) and project to the contralateral DLPFC (39, 40), and both regions are implicated in ASD pathology (41). We hypothesized that Glx levels in the LDLPFC and the R cerebellum will be predictive of changes in scores on social assessments after 12 weeks of treatment with memantine in a sample of 10 adult participants with ASD. Specifically, we expected that those with the highest Glx in these regions of interest would be the best responders on the social domain for CGI-I. In addition to the primary hypotheses concerning Glx, we examined other neurometabolites assessing neuronal health, viability, and quantity - N-acetylaspartate (NAA) (34), glial integrity (higher levels reflecting astroglial activation, gliosis, and inflammation) - myo-inositol (Ins) (35), and brain energy metabolism (creatine is well-established for its role in energy metabolism, and phosphocreatine is a potent antioxidant) - creatine and phosphocreatine (Cr+PCr) (36), to explore how they might be involved in treatment response and whether models that incorporate multiple neurometabolites may account for more variation in treatment response (42).

METHODS

Participants

Adolescent and adult patients with a confirmed diagnosis of ASD and who were willing to try memantine as an off-label clinical follow-on treatment were recruited through clinics at the Thompson Center for Autism and Neurodevelopmental Disorders, University of Missouri, Columbia, Missouri. General inclusion criteria were: (1) Age $\geq$ 16 years, (2) ASD diagnosis as per DSM V determined by clinician interview and confirmed with an Autism Diagnostic Interview-Revised (ADI-R) or Autism Diagnostic Observation Schedule (ADOS), and (3) Score <4 on the Clinical Global Impressions – Severity (CGI-S) scale indicating mild to moderate illness. The mild to moderate illness group was selected to ensure patient comfort and safety while taking the exploratory nature of the study into consideration. Exclusion criteria were: (1) Contraindications to MRI (Magnetic Resonance Imaging) (e.g., metallic implants, pacemakers, claustrophobia, pregnancy, lactation), (2) memantine intolerance or known hypersensitivity to memantine hydrochloride or to any components used in the formulation, and (3) medications that might interact with memantine. All procedures were approved by the University of Missouri Institutional Review Board and all participants (and legal guardians, for participants <18 years of age) provided written consent/assent, as applicable.

Measures

At baseline, participants were assessed on the following social and behavioral measures:

The Clinical Global Impressions – Severity (CGI-S) is a clinician rated scale (range one to seven, with one being no symptoms and seven being the most severe symptoms possible) to assess severity of symptoms, such as, social interaction, sensory sensitivities, restricted interests, verbal and non-verbal communication, etc. and is commonly used in ASD research (19, 21, 22). The CGI-S for social behavior was the focus in this study.

The Social Responsiveness Scale (SRS) is a well-validated 65 item questionnaire that specifically evaluates social deficits associated with ASD. Several studies in ASD have used SRS to track social outcomes in response to pharmacological interventions (21–29).

Imaging

Following baseline clinical assessments, subjects underwent an MRI scan, including structural MRI and ^{1}H-MRS on a Siemens 3-Tesla TIM Trio MRI scanner located in the Brain Imaging Center at the University of Missouri. Participants were asked not to consume any forms of caffeine or alcohol 8 h before the scanning to eliminate effects from caffeine/alcohol on neurometabolites. High-resolution T1-weighted structural images were acquired using the three-dimensional multiplanar rapidly acquired gradient echo (MP-RAGE) pulse sequence: repetition time (TR), 2,500 ms; echo time (TE), 438 ms; flip angle, 8°, 256 × 256 voxel matrix; field of view (FOV), 256 mm; 176 axial slices; thickness, 1 mm. These images were used to quantify the brain tissue composition within the spectroscopic voxel and exclude any pathology. Based on anatomical landmarks, single voxel spectroscopy (SVS, 2 × 2 × 2 cm^3) with Point RESolved Spectroscopy (PRESS, TE = 80 ms, TR = 2,000 ms, 128 averages, flip angle=90°, water suppression bandwidth = 50 Hz, delta frequency = −2.3 ppm, bandwidth = 1,200 Hz) was prescribed to the right posterolateral hemisphere of the L cerebellum targeting crus I/11 and the LDLPFC based on frontal gyral markers (**Figure 1**), brain regions previously identified as revealing changes in Glx/GABA and connectivity associated with performance on social communication (32). The same trained research personnel (NN) positioned the voxels on all the participants during the scanning sessions. Levels of Glx and other

metabolites (NAA, Ins, and Cr+PCr) were examined. To avoid lipid artifact from the skull, six outer voxel suppression saturation bands were applied around the SVS. Automated, followed by manual, shimming was performed to achieve an optimal full width at half maximum of <20 Hz of the water signal from the entire excitation volume. Internal reference water signal was also acquired by using non–water suppressed MRS imaging to calculate absolute concentrations of neurometabolites of interest.

Memantine Administration

Following the baseline imaging and behavioral assessment, participants were administered memantine, starting at 5 mg/day doses, and titrated up over 28 days to 20 mg/day based on response and tolerability, for a period of 12 weeks.

Follow-Up Assessments

Upon completion of the 12 weeks of memantine, the participants repeated clinical assessments using the CGI-S for social interaction and SRS. The CGI-I for social interaction (ratings from 1 to 7, with 4 being no change, and decreasing scores representing minimal (3), marked (2) and dramatic (1) improvement, and increasing scores similarly representing worsening) (19, 21, 22) was also assessed at this time point and the social interaction subscale of the CGI-I served as the primary outcome measure.

Analyses

Absolute concentrations of each metabolite– Glx, NAA, Ins, and Cr+PCr – measured in our regions of interest (ROI): LDLPFC and R cerebellum were quantified using Linear Combination of Model Spectra (LCModel) software [V6.3 (30)] with a standard PRESS basis set and water as internal concentration reference (31). The metabolites quantified in this manner serve as an estimate of their concentrations within the examined ROI (30, 31). Each metabolite of interest was expressed in institutional units (IU; ∼ millimoles per kilogram wet weight) for each ROI. Gray matter volume within spectroscopy voxel was quantified using SPM (Mathworks Inc.) and controlled for during the statistical analysis. Processing of spectroscopic data is described in detail in our previous work (32).

For this pilot study, we summarized variables (primary: Glx; secondary: NAA, Ins, Cr+PCr) and outcomes (primary: scores on CGI-I for social interaction; secondary: SRS) measures by mean and standard deviation. To address our main hypothesis, responders and nonresponders were compared for Glx (and other neurometabolites) concentrations in each ROI using independent samples *t*-test. Pearson correlation analysis was also used to examine the relationships between Glx in each ROI and (1) scores on the social interaction subscale of the CGI-I social post-treatment and (2) changes in scores on the SRS baseline vs. post-treatment. Stepwise linear hierarchical regression models were used to examine whether baseline concentrations of neurometabolites (Glx, NAA, Ins, and Cr+PCr) in the LDLPFC and the R cerebellum predicted changes in scores on outcome measures using SPSS (IBM Corp, v26).

RESULTS

Ten participants (mean ± SD age = 24 ± 4 years, range 17–32 years old, one female, all Caucasian) were recruited to be a part of the study through the Thompson Center for Autism and Neurodevelopmental Disorders, University of Missouri, Columbia, Missouri. CGI-I scores on the social interaction subscale post 12 weeks of treatment with memantine were used to classify the participants into responders (scores 1–3, $n = 5$) and non-responders (scores 4–7, $n = 3$) (**Figure 2**).

Two subjects dropped out of the study at week 2 on memantine due to worsening behavioral symptoms, and were included in the non-responders group, as a result, for a total of $n = 5$ non-responders. No other side effects were reported.

All neurometabolites included in the analyses were within the limits required for spectra to be of acceptable quality: %SD <25 (%SD or Cramer Rao lower bounds, representing the threshold of the error associated with model fitting) and signal to noise >10. Due to head motion-related artifacts data from one participant had to be dropped from further analysis. Three other participants had high lipid contamination in the spectra acquired from the LDLPFC, suggesting tissue other than brain was included, and were not included in the corresponding models.

Overall, there were $n = 9$ participants with pre- and $n = 7$ participants with post-trial outcome data, of which $n = 6$ had high quality MRS data from the LDLPFC and $n = 9$ for the R cerebellum.

Comparison of Responders and Non-responders

When responders and non-responders were compared, with the two participants dropping out due to worsening symptoms categorized as non-responders, no significant differences were observed for either of the two defined ROIs for Glx (LDLPFC: responders 7.00 ± 0.87 IU, non-responders 5.11 ± 1.47 IU, $t = -2.08$, $p = 0.10$ (see **Figure 3A**); R cerebellum: responders: 7.95 ± 2.21 IU, non-responders: 6.86 ± 2.86 IU, $t = -0.60$, $p = 0.57$). However, the number of participants with high quality data in the non-responder group was very limited for the LDLPFC, but if the *p*-value is to be believed, a larger sample size may reach statistical significance.

Our series of analysis of the secondary neurometabolites showed significantly higher levels of NAA in the LDLPFC in responders compared to the non-responders (9.78 ± 0.71 IU vs. 6.61 ± 1.65 IU, $t = -3.56$, $p = 0.024$) (see **Figure 3B**), again with a very limited number of non-responders with high quality data in the LDLPFC. A similar pattern, increased levels in responders vs. non-responders, was also observed for Cr+PCr levels in the R cerebellum; however, this difference did not reach statistical significance (responders: 12.34 ± 1.01 IU, non-responders 10.30 ± 1.35 IU, $t = -2.13$; $p = 0.10$).

Correlations

Partial correlations controlling for age and gray matter volume fraction within ROI revealed that CGI-I scores on the social interaction subscale posttreatment were not directly correlated with Glx (LDLPFC: $r = -0.76$, $p = 0.24$

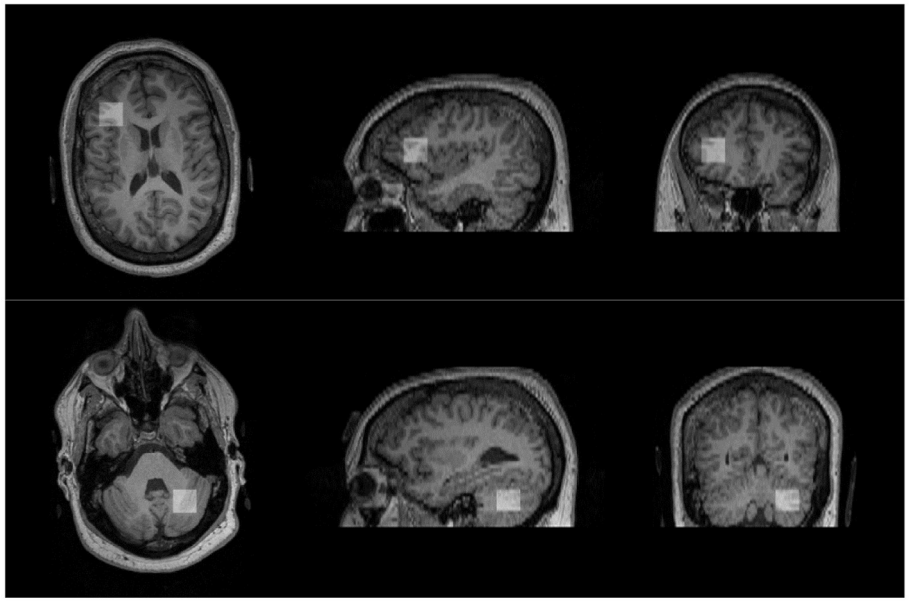

FIGURE 1 | Approximate positions of voxels placed in the regions of interest. Voxel of interest (indicated by white square) placed in the left dorsolateral prefrontal cortex (top) and the right cerebellum (bottom).

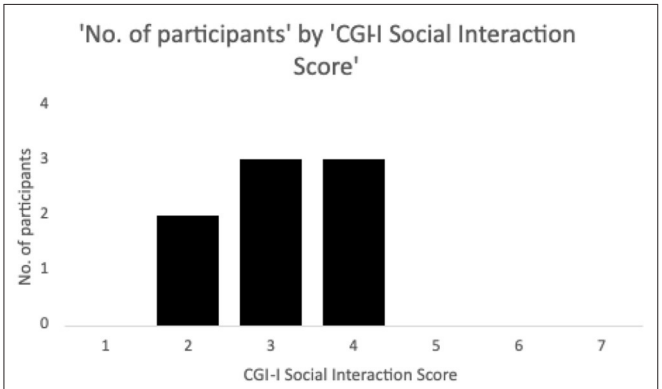

FIGURE 2 | Number of participants by Clinical Global Impressions – Improvement (CGI-I) social interaction scores following 12 weeks of memantine treatment. Ratings on the scale range from 1 to 7, with 4 being no change, and decreasing scores representing minimal (3), marked (2) and dramatic (1) improvement, and increasing scores similarly representing worsening. Patients with scores 1–3 on the social interaction subscale were classified as responders while scores of 4–7 were considered non-responders. Two additional participants who discontinued the drug due to increased behaviors and did not complete the CGI-I were classed as non-responders but not shown in the Figure.

(see **Figure 3C**); R cerebellum: $r = -0.45$; $p = 0.55$ (see **Figure 3D**) or other neurometabolite levels at baseline in either of the two ROIs ($p > 0.1$ for all). The change in SRS score (calculated based on subscale T-scores, baseline vs. posttreatment) was negatively associated with baseline Cr+PCr levels in the LDLPFC ($r = -0.956$, $p = 0.04$) (see **Figure 3E**).

Hierarchical Regression Models

Linear hierarchical regression models revealed that a final model with Glx ($B = -1.07$, $p = 0.02$) and Ins ($B = 0.58$, $p = 0.04$) in the LDLPFC at baseline significantly predicted scores on the social interaction CGI-I posttreatment (R^2 adjusted $= 0.86$, $F = 9.188$, $p = 0.05$).

DISCUSSION

This pilot study examined whether ^{1}H-MRS-detected Glx (and other neurometabolites) in two targeted brain regions was associated with or could predict treatment response to a moderate affinity NMDA receptor antagonist, memantine, in adults and adolescents with ASD. Five participants did respond (scores of 1–3 on the CGI-I for social interaction) while five did not respond (scores of 4–7, or stopped due to increased behaviors) to memantine. Our preliminary data did not reveal a relationship between baseline Glx levels in either ROI and response to memantine. However, Glx along with Ins within the LDLPFC was found to predict the post-treatment scores on the social interaction subscale of the CGI-I. Specifically, it appears that higher levels of Glx and lower levels of Ins in the LDLPFC were predictive of lower scores on the CGI-I (or greater improvement) following treatment with memantine.

Additionally, higher levels of Cr+PCr in the LDLPFC at baseline were associated with decreases in SRS total score post treatment. Higher NAA levels were also found in the LDLPFC in responders than in non-responders. Since creatine levels reflect cellular energy metabolism and NAA levels are connected to energy metabolism in neuronal mitochondria, these results may indicate that the effectiveness of memantine treatment

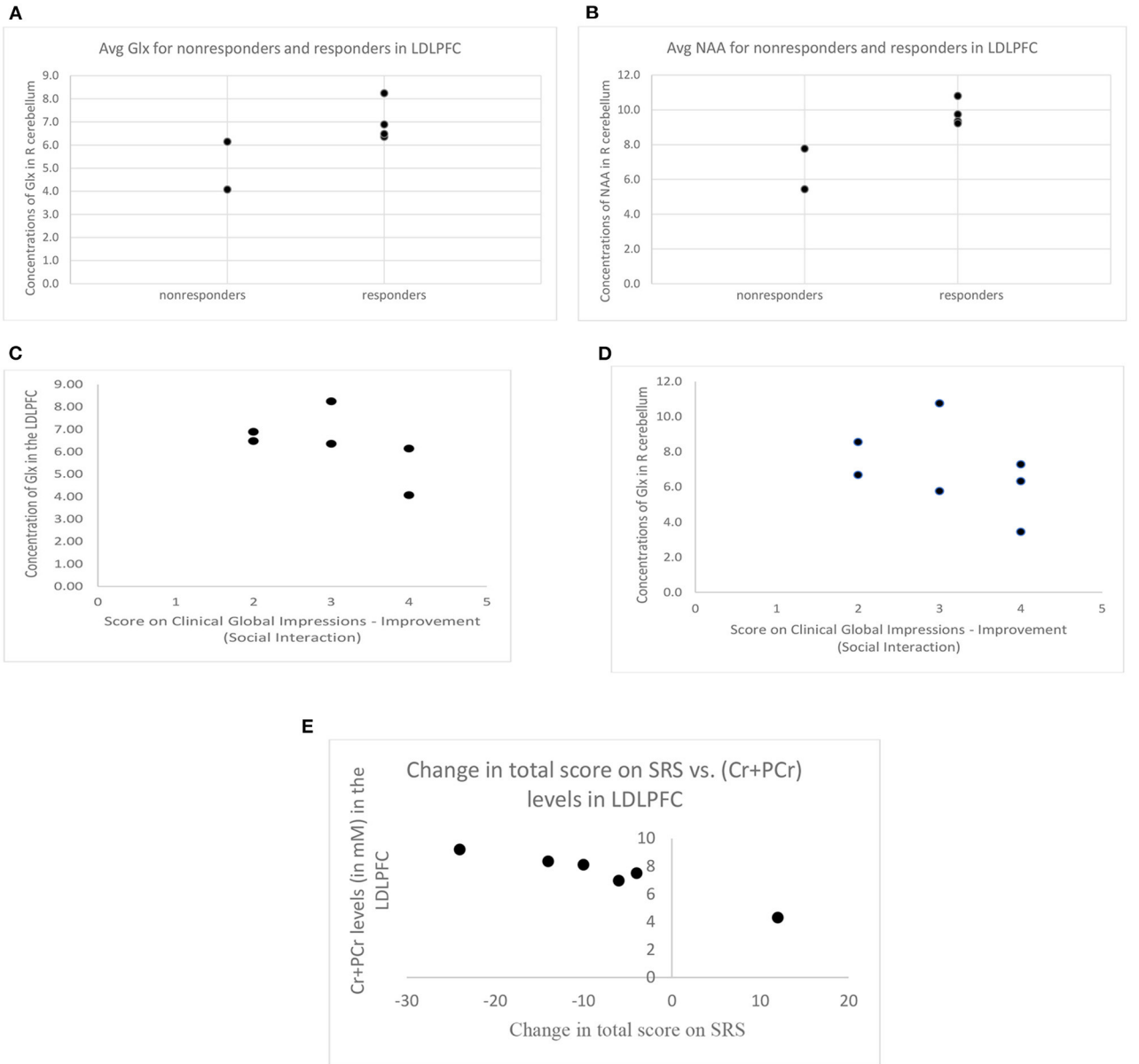

FIGURE 3 | (A) Difference in Glx concentration between responders (CGI-I score of 3 or less) and non-responders (CGI-I score of 4 or more, or withdrew due to worsening behaviors) and **(B)** difference in NAA concentration between responders and non-responders (error bars represent standard deviation, * = $p < 0.05$, #= $p \leq 0.1$). **(C)** Concentration of Glx in the LDLPFC and **(D)** concentration of Glx in the R cerebellum across CGI-I scores for all participants that do have follow-up visits allowing the obtaining of CGI-I scores. **(E)** Change in SRS total score (negative values indicate improvement posttreatment) was negatively associated with (Cr+PCr) levels in the LDLPFC. SRS, Social Responsiveness Scale. LDLPFC, Left dorsolateral prefrontal cortex. Glx, Glutamate and its precursor glutamine; LDLPFC, Left dorsolateral prefrontal cortex; R cerebellum, right cerebellum; Cr+PCr, Creatine + Phosphocreatine; SRS, Social Responsiveness Scale; CGI-I, Clinical Global Impressions-Improvement.

was dependent on the level of mitochondrial dysfunction, a phenomenon commonly noted in ASD pathology (33), in the LDLPFC. However, these results will need confirmation in larger samples. Additionally, larger samples will allow the possibility of understanding how factors such as head circumference and intellectual functioning might relate to these findings.

These preliminary results are an initial effort to understand the mixed results reported in previous studies using memantine in ASD. It is possible that there exist different subsets of autism that respond differently to this treatment. Our hypothesis was that glutamatergic levels would predict response. However, the preliminary evidence suggests that there may be a more complex relationship, where increased glutamatergic levels, in the additional setting of altered glial and cellular energy metabolism markers, in the LDLPFC may show more improvements with memantine. Additionally, when responders were compared to

non-responders (allowing inclusion of participants that withdrew due to not tolerating the medication), NAA levels in the LDLPFC differed between groups, providing further support that brain energy metabolism and neuronal integrity are important in predicting therapy response. Overall, these preliminary results raise the possibility of using 1H-MRS as a tool to discover potential biomarkers for treatment response in ASD. However, larger sample sizes will be needed to confirm these findings, and additionally to determine whether significant effects might be revealed for the relationship between Glx (and other neurometabolites) and treatment response with a more robust sample. For instance, a weak trend was observed for greater LDLPFC Glx among the good responders ($p = 0.1$). Based on these data, if the results from such a small sample are to hold true in further study, a sample size of 11 per group (responders, non-responders) would be sufficient for a power of 0.80 to yield a significant group difference in LDLPFC Glx at $\alpha = 0.05$. Additionally, other neurometabolites, e.g., GABA, may be relevant, given the recent work demonstrating relationships between functional connectivity an excitatory/inhibitory balance in ASD (5, 32). While the sample size is small for extensive interpretation of these findings, future studies can explore whether response in the social domain to memantine might be related to glial function, as may be suggested by the relationship with Ins, whereby response is greatest with increased glutamate specifically in the setting of less activated microglia. The direction of this outcome is unexpected, as we would have predicted that patients with more activated microglia would have an augmented response to the inhibition of excitatory activity with an NMDA antagonist in the setting of increased baseline glutamate, do to putative compounding of the hyperexcitable state, so this further highlights the need for confirmation in future studies.

This line of work could, in turn, have important implications for clinical care including improving accuracy of individual prognosis and individualizing treatments in ASD. Recent studies have suggested that memantine might better target cognitive outcomes in ASD rather than social (18). However, the findings from the current study begin to raise the question as to whether social outcomes might still be relevant in an optimally targeted subset of patients.

The small sample size (particularly for 1H-MRS analysis) is a definite limitation of the current exploratory pilot study. This impacts the generalizability of our findings. Additionally, selection of patients capable of participating in the imaging session without sedation likely also introduces a bias in the findings, further impacting generalizability. Future studies should, therefore, be performed with larger samples. Additionally, while our study specifically targeted Glx since the drug memantine targets glutamate receptors, it would be critical for future work to gain a better understanding of the more complete role of the balance in excitation/inhibition in predicting the effects of memantine or related agents, including targeting GABA. Expanding the regions of interest to include other regions within the networks involved in social communication would also be critical in future studies, in addition to incorporating newer automated techniques for ROI optimization to improve outcomes.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by University of Missouri. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

DB, JH, and NN designed the project. NN implemented the work with DB. CC oversaw the MRS aspects throughout. MG also helped guide the MRS analysis. CA helped to retrieve and prepare the data from the database in preparation for the analysis by MG. All authors contributed to the article and approved the submitted version.

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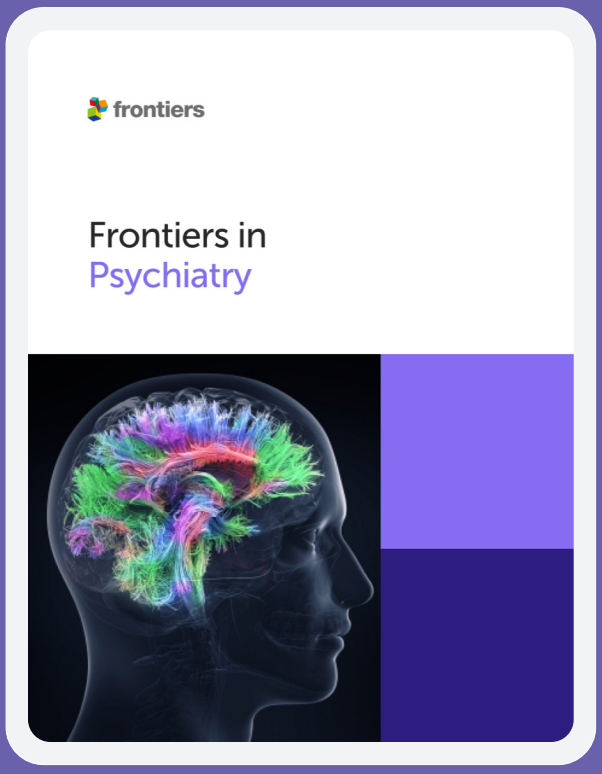

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