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Saliva MicroRNA Differentiates Children With Autism From Peers With Typical and Atypical Development

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Objective: Clinical diagnosis of autism spectrum disorder (ASD) relies on time-consuming subjective assessments. The primary purpose of this study was to investigate the utility of salivary microRNAs for differentiating children with ASD from peers with typical development (TD) and non-autism developmental delay (DD). The secondary purpose was to explore microRNA patterns among ASD phenotypes.

Method: This multicenter, prospective, case-control study enrolled 443 children (2–6 years old). ASD diagnoses were based on *DSM-5* criteria. Children with ASD or DD were assessed with the Autism Diagnostic Observation Schedule II and Vineland Adaptive Behavior Scales II. MicroRNAs were measured with high-throughput sequencing. Differential expression of microRNAs was compared among the ASD (n = 187), TD (n = 125), and DD (n = 69) groups in the training set (n = 381). Multivariate logistic regression defined a panel of microRNAs that differentiated children with ASD and those without ASD. The algorithm was tested in a prospectively collected naïve set of 62 samples (ASD, n = 37; TD, n = 8; DD, n = 17). Relations between microRNA levels and ASD phenotypes were explored.

Result: Fourteen microRNAs displayed differential expression (false discovery rate < 0.05) among ASD, TD, and DD groups. A panel of 4 microRNAs (controlling for medical/demographic covariates) best differentiated children with ASD from children without ASD in training (area under the curve = 0.725) and validation (area under the curve = 0.694) sets. Eight microRNAs were associated ($R > 0.25$, false discovery rate < 0.05) with social affect, and 10 microRNAs were associated with restricted/repetitive behavior.

Conclusion: Salivary microRNAs are “altered” in children with ASD and associated with levels of ASD behaviors. Salivary microRNA collection is noninvasive, identifying ASD-status with moderate accuracy. A multi-“omic” approach using additional RNA families could improve accuracy, leading to clinical application.

Clinical trial registration information: A Salivary miRNA Diagnostic Test for Autism; https://clinicaltrials.gov/; NCT02832557.

Key words: autism, microRNA, diagnosis, biomarker, saliva

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Autism spectrum disorder (ASD) represents a continuum of deficits in communication and social interaction and restrictive, repetitive interests and behaviors. Health care providers have an opportunity to improve outcomes for children with ASD through early diagnosis and referral for evidence-based behavioral therapy.[1,2] Studies suggest earlier treatment contributes to improved social and behavioral outcomes.

An important barrier in the evaluation and treatment of ASD is the lack of objective assessment tools.[3-5] Recognition of ASD symptoms generally occurs no earlier than 18 to 24 months of age, when deficits in communication emerge.[6] Screening at this stage typically relies on the Modified Checklist for Autism in Toddlers–Revised (MCHAT-R). This parental survey is less than 50% specific.[7] In 2017 the US Preventive Services Task Force determined that insufficient evidence existed to recommend ASD screening.[8] Nonetheless, the American Academy of Pediatrics continues to advocate for universal ASD screening, and pediatricians, faced with no alternative, continue to use subjective, nonspecific tools. Clearly, a more accurate and objective toolset would improve ASD evaluation and therapy.

Given the multifactorial genetic and environmental risk factors that have been identified in ASD, it is possible that at least 1 epigenetic mechanism might play a role in ASD pathogenesis.[9] Among these potential mechanisms are microRNAs (miRNAs). MiRNAs are non-coding nucleic acids that can regulate expression of entire gene networks by repressing the transcription of mRNA into proteins, or

promoting the degradation of target mRNAs.[10] MiRNAs are essential for normal brain development and function. Notably, miRNAs can be packaged within exosomes and other lipophilic carriers as a means of extracellular signaling. This feature allows noninvasive measurement of miRNA levels in extracellular biofluids such as saliva[11] and renders them attractive biomarker candidates for disorders of the CNS.[12]

Studies of miRNA in children with ASD have demonstrated differential expression patterns in postmortem brain tissue,[13,14] serum, and cultured peripheral lymphoblasts.[15,16] Several miRNAs identified in these studies target genes known to be involved in ASD pathogenesis.[17] Brain biopsy is clearly too invasive to be suitable for ASD screening and the physiologic relevance of miRNA expression in cultured lymphoblasts introduces methodologic concerns. Given the robust cranial nerve innervation of the oropharynx, its proximity to glymphatic structures, and the sensorimotor pathology observed in children with ASD (food texture sensitivity,[18] taste aversions, and speech apraxia[19]), we previously explored the potential of salivary miRNA to differentiate children with ASD from typically developing peers.[20] A pilot study of 24 children with ASD demonstrated that salivary miRNAs are altered in ASD and broadly correlate with miRNAs reported to be altered in the brains of children with ASD.

Together, these studies support the potential utility of miRNA measurement in ASD screening. However, the clinical applicability of miRNA studies in persons with ASD has been limited by several factors: no miRNA study has used more than 55 participants with ASD,[21] despite the broad, heterogeneous nature of the disorder; no miRNA study has enrolled children at the ages (2–6 years) when ASD diagnosis first occurs (ie, when a diagnostic biomarker panel would have the most clinical utility); no miRNA study has compared children with ASD to peers with non-autism developmental delay (DD)—a comparison required to develop a robust diagnostic toolset; and no study has examined the ability of miRNA signatures to differentiate ASD phenotypes, a priority for the autism community.

The present study sought to address these deficiencies in the literature and establish the diagnostic utility of salivary miRNAs in ASD. We hypothesized that characterization of salivary miRNA concentrations in children with ASD, DD, and typical development (TD) would identify a panel of miRNAs with diagnostic potential. We posited that these miRNAs would exhibit brain-related targets on functional pathway analyses and display associations with specific autism phenotypes (assessed through standard measures of communication, socialization, and repetitive behavior).

METHOD

Ethical approval for this study was obtained from the institutional review boards at the Penn State College of Medicine (Hershey) and the State University of New York (SUNY) Upstate Medical University (Syracuse). Written informed consent was obtained from the parent/caregiver of each participant.

Participants

This multicenter, cross-sectional, prospective, case-control study included 443 children 2 to 6 years old receiving well-child or developmental specialist care at the Penn State College of Medicine or SUNY Upstate Medical University. The 2- to 6-year age group was chosen to include children at the earliest ages of ASD diagnosis, when screening and diagnostic biomarkers would be of most clinical benefit. Recruitment occurred at academic, outpatient, and primary and tertiary care clinics from October 2015 through April 2018. In the training set (used for miRNA exploration and creation of the regression algorithm), there were 187 children with ASD, 125 children with TD, and 69 children with DD. In the prospective test set (used for validation of the regression algorithm), there were 37 children with ASD, 8 children with TD, and 17 children with DD. Nearly equal numbers of participants with ASD, TD, and DD were recruited from each site. An a priori analysis using Power Analysis and Sample Size Software (version 15; NCSS, LLC, Kaysville, UT) and setting the null area under the curve (AUC) to 0.7, determined that the sample size used in the training set provided 85% power to detect an AUC equal to 0.77 (based on 1-sided z test with $\alpha = .05$) and 99% power to detect an AUC greater than 0.8. Similarly, the replication cohort ($n = 62$) had 85.6% power to detect an AUC equal to 0.78 when comparing children with ASD with those without ASD. ASD status was defined by *DSM-5* diagnosis, confirmed by physician assessment within the previous 12 months, and supported by evaluation with the Autism Diagnostic Observation Schedule II (ADOS-II; or other standardized assessment tool such as the Checklist for Autism Spectrum Disorder, the Autism Diagnostic Interview–Revised, or the Childhood Autism Rating Scale). TD status was defined by a history of negative ASD screening on the MCHAT-R and documentation of typical development at a pediatric well-child visit within the previous 12 months. DD status was defined by a clinical deficit in gross motor, fine motor, expressive communication, receptive communication, or socialization that was identified by standardized screening (Survey of Wellbeing in Young Children, MCHAT-R, or Parents Evaluation of Developmental Status) at a regularly scheduled visit, but not

meeting *DSM-5* criteria for ASD. Targeted recruitment was used to match age and sex across ASD, DD, and TD groups. Exclusion criteria for all groups included feeding-tube dependence, active periodontal disease, upper respiratory infection, fever, confounding neurologic (ie, cerebral palsy, epilepsy) or sensory (ie, blindness, deafness) impairment, and wards of the state. Participants with TD and a medical condition requiring daily medication or pediatric specialist care also were excluded.

Participant Characterization

For all participants, extensive medical and demographic characterization was performed, including age, sex, ethnicity, birth age, birth weight, perinatal complications, current weight, body mass index, oropharyngeal status (eg, allergic rhinitis), dietary restrictions, medications, chronic medical issues, immunization status, medical allergies, early intervention services, surgical history, and family psychiatric history. Given the prevalence of attention-deficit/hyperactivity disorder (ADHD)[22] and gastrointestinal (GI) disturbance[23] in children with ASD, survey questions were included to identify these 2 common medical comorbidities. GI disturbance was defined by the presence of constipation, diarrhea, abdominal pain, or reflux on parental report, *International Statistical Classification of Diseases and Related Health Problems, Tenth Revision* (*ICD-10*) chart review, or use of stool softeners/laxatives in the child's medication list. ADHD was defined by parental report or *ICD-10* chart review. Adaptive skills in communication, socialization, and daily living activities were measured in all participants using the Vineland Adaptive Behavior Scale II (VABS-II) and standardized scores were reported. Evaluation of ASD symptomology (ADOS-II) was completed when possible for participants with ASD and DD (n = 164). Social affect, restricted repetitive behavior, and ADOS-II total scores were recorded.

Saliva Collection and RNA Processing

Saliva was collected from all children in a nonfasting state using a P-157 Nucleic Acid Stabilizing Swab (DNA Genotek, Ottawa, ON, Canada). Saliva was obtained from the sublingual and parotid regions of the oral cavity over a 5- to 10-second period, taking care to avoid the teeth when possible (https://www.youtube.com/watch?v=AzCpHWqhRQs&feature=youtu.be). Time of saliva collection was recorded, and swabs were kept at room temperature in stabilization solution for up to 4 weeks before storage at −20°C. Salivary miRNA was purified using a standard Trizol method, followed by a second purification with an RNeasy mini column (Qiagen, Germantown, MD). The yield and quality of RNA samples were assessed using the Agilent Bioanalyzer (Agilent, Technologies, Santa Clara, CA) before library construction. RNA was sequenced at the SUNY Molecular Analysis Core at Upstate Medical University with an Illumina TruSeq Small RNA Sample Prep protocol (Illumina; San Diego, CA). The targeted read depth for each sample was 10 million reads using 50 base-pair single-end reads on a NextSeq500 instrument (Illumina). Reads for each sample were aligned to the hg38 build of the human genome in Partek Flow (Partek, St. Louis, MO) with the SHRiMP2 aligner. Total miRNA counts within each sample were quantified with miRBase precursor- and mature-microRNA v21. Poor-quality reads (mean q score < 30) were eliminated, and samples with total mature miRNA read counts less than 20,000 were excluded. Of the 2,813 mature miRNAs aligned, we interrogated 527 miRNAs for differential expression among groups. The 527 miRNAs included those with robust expression (raw read counts >10 in $\geq 10\%$ of samples; 375 miRNAs) and those identified in previous ASD studies[17] and detectable in saliva (raw counts >1 in 10% of samples; 152 miRNAs). Before statistical analysis, read counts were quantile-normalized, mean-centered, and divided by the standard deviation of each variable.

Statistical Analyses

The primary outcome of this study was the identification of miRNAs that could differentiate children with ASD from children without ASD (including those with TD and DD) via logistic regression analysis. Differences in medical and demographic characteristics between groups were compared using 2-tailed Student t test. In the training set (n = 381), a nonparametric Kruskal-Wallis test and a partial least squared discriminant analysis (PLS-DA) were used to identify individual miRNA candidates for differentiating children with ASD from peers with TD and DD. The miRNAs with significant differences between groups (false discovery rate [FDR] < 0.05) and/or PLS-DA weighted sum of absolute regression coefficients of at least 2.0 were selected for biomarker testing. To control for confounding, medical and demographic characteristics were included in the logistic regression analysis as covariates. In addition, we explored the potential influence of RNA quality on any significant miRNA variables using analysis of covariance with Diagnosis and RNA Integrity Number (RIN) and their interaction used as main and interaction effects, respectively. Biomarker exploration was performed with Metaboanalyst R package (McGill University, Montreal, QC, Canada; http://www.metaboanalyst.ca/faces/ModuleView.xhtml) using the

biomarker workflow.[24] The training set was used to determine threshold (cutoff) concentrations for miRNAs, which were used in ratios with selected medical/demographic covariates. To avoid "overfitting" the model and to ensure that the miRNAs accurately differentiated participants with ASD, the algorithm was tested in a naïve replication set of 62 children. Performance was evaluated using AUC analysis from receiver operating characteristic curves generated in the training and test sets.

Associations between salivary miRNA concentrations and ASD phenotypic characteristics were explored with Spearman rank correlations (for dichotomous variables) or Pearson correlations (for continuous variables), with FDR correction (FDR < 0.05). The phenotypic characteristics of interest included adaptive behavior scores (VABS-II); ASD traits (ADOS-II scores); and medical comorbidities (presence/absence of GI disturbance or ADHD). Relations between salivary miRNA concentrations and confounding medical/demographic characteristics (ie, age, sex, ethnicity, body mass index, asthma, allergic rhinitis, time of collection, time of last meal, dietary restrictions) also were evaluated with Pearson or Spearman rank correlations. Any miRNA-variable association in which R was greater than 0.25 and FDR was less 0.05 was reported as significant.

Secondary analyses investigated the mRNA targets for 2 sets of miRNAs: the miRNAs "altered" between ASD, TD, and DD groups based on initial Kruskal-Wallis testing and the miRNAs associated with ASD features at ADOS testing. For the latter, we also used multivariate regression to adjust the correlations by the RIN value and RNA sequencing quality (Q) scores. Functional analysis was performed for each miRNA set in DIANA mirPath v3 online software (http://snf-515788.vm.okeanos.grnet.gr/).[25] The microT-cds algorithm was used to identify species-specific gene targets for each miRNA. DIANA mirPath identified Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways with significant (FDR < 0.05) target enrichment using Fisher exact test. A list of high-confidence mRNA targets (experimentally validated miRNA-mRNA interaction with microT-cds score ≥ 0.975) was interrogated for protein-protein interaction networks using moderate stringency settings (interaction score > 0.40) in String 10 software (http://string-db.org).[26] Enrichment of mRNA target lists for the 961 autism-associated genes in the SFARI autism database (https://gene.sfari.org/database/human-gene/)[27] was explored using χ^2 test with Yates correction. The number of overlapping mRNAs was reported, in addition to enrichment relative to a random sampling of the approximately 20,000 coding mRNAs.

RESULTS

Participant Characteristics

Two-tailed Student t tests were used to compare demographic and medical characteristics among ASD, TD, and DD groups in the training set (Table 1A) and test set (Table 1B). In the training set, the average age of participants with ASD (54 $\pm$ 15 months) was older ($p = .006$) than that of participants with TD (47 $\pm$ 18 months) but not of participants with DD (50 $\pm$ 13 months; $p = .076$). The ASD group had a larger proportion of boys (161 of 187; 86%) than the TD group (76 of 126; 60%; $p = 1.0\text{E-}6$) and the DD group (48 of 69; 70%; $p = .015$). Children with ASD had higher rates of GI disturbance (35 of 187; 19%) than children with TD (2 of 125; 2%; $p = 5.4\text{E-}7$), but not children with DD (13 of 69; 19%; $p = 0.92$). The ASD group also had higher rates of ADHD (43 of 187; 25%) than the TD group (10 of 125; 8%; $p = .0003$), but not the DD group (21 of 69; 30%; $p = 0.26$). There were no significant differences ($p < .05$) among the 3 groups in the proportion of Caucasian children (274 of 381; 72%), average body mass index (18.9 $\pm$ 11 kg/m^2), rates of asthma (43 of 381; 11%) or allergic rhinitis (81 of 381; 21%), time of saliva collection (13:00 $\pm$ 3 hours), or rates of dietary restrictions (50 of 381; 13%).

In the test set, children with ASD had higher rates of asthma (4 of 37; 11%; $p = .044$) and ADHD (6 of 37; 16%; $p = .012$) compared with peers with TD or DD. There were higher rates of allergic rhinitis in children with ASD (5 of 37; 14%) relative to children with TD (0 of 8; 0%; $p = .023$). There was no difference among the ASD, TD, and DD groups in mean age (47 $\pm$ 14 months), proportion of boys (49 of 62; 79%), mean body mass index (17.5 $\pm$ 4 kg/m^2), or rates of GI disturbance (12 of 62; 19%).

Neuropsychiatric characteristics were assessed with the VABS-II (adaptive behaviors; ASD, TD, and DD groups) and the ADOS-II (ASD features; ASD and DD groups only). Standard scores were compared among groups using 2-tailed Student t tests. In the training set, children with ASD had lower standardized communication scores (73 $\pm$ 20) than children with TD (103 $\pm$ 17; $p = 3.5\text{E-}27$) or DD (79 $\pm$ 17; $p = .044$). The ASD group also had lower mean scores in socialization (73 $\pm$ 15) and activities of daily living (75 $\pm$ 15) than the TD group (socialization = 108 $\pm$ 18; $p = 2.0\text{E-}33$; activities of daily living = 103 $\pm$ 15, $p = 1.7\text{E-}29$) and the DD group (socialization = 82 $\pm$ 20; $p = .006$; activities of daily living = 83 $\pm$ 19; $p = .009$). Children with ASD had higher mean scores on the social affect (13 $\pm$ 5) and restricted/repetitive behavior (3 $\pm$ 2) components of the ADOS-II than did counterparts with

TABLE 1 Participant Characteristics

A. Training Set

Characteristic	All Groups (n = 381)	ASD (n = 187)	TD (n = 125)	DD (n = 69)
Demographics and anthropometrics				
Age (mo), mean (SD)	51 (16)	54 (15)	47 (18)*	50 (13)
Boys, n (%)	285 (75)	161 (86)	76 (60)*	48 (70)*
Caucasian, n (%)	274 (72)	132 (71)	95 (76)	47 (69)
BMI (kg/m^2), mean (SD)	18.9 (11)	17.2 (7)	21.2 (16)	19.5 (10)
Clinical characteristics				
Asthma, n (%)	43 (11)	19 (10)	10 (8)	14 (20)
GI disturbance, n (%)	50 (13)	35 (19)	2 (2)*	13 (19)
ADHD, n (%)	74 (19)	43 (23)	10 (8)*	21 (30)
Allergic rhinitis, n (%)	81 (21)	47 (25)	19 (15)	15 (22)
Time of collection (h), mean (SD)	13:00 (3)	13:00 (3)	13:00 (2)	13:00 (3)
Time since last meal (h), mean (SD)	2.8 (2.5)	2.9 (2.5)	3.0 (2.9)	2.1 (1.1)*
Dietary restrictions, n (%)	50 (13)	28 (15)	10 (8)	12 (18)
Neuropsychiatric factors				
Communication by VABS-II standard score, mean (SD)	83 (23)	73 (20)	103 (17)*	79 (18)*
Socialization by VABS-II standard score, mean (SD)	85 (23)	73 (15)	108 (18)*	82 (20)*
Activities of daily living by VABS-II standard score, mean (SD)	85 (20)	75 (15)	103 (15)	83 (19)*
Social affect by ADOS-II score, mean (SD)	—	13 (5)	—	5 (3)*
Restrictive/repetitive behavior by ADOS-II score, mean (SD)	—	3 (2)	—	1 (1)*
ADOS-II total score, mean (SD)	—	16 (6)	—	6 (4)*

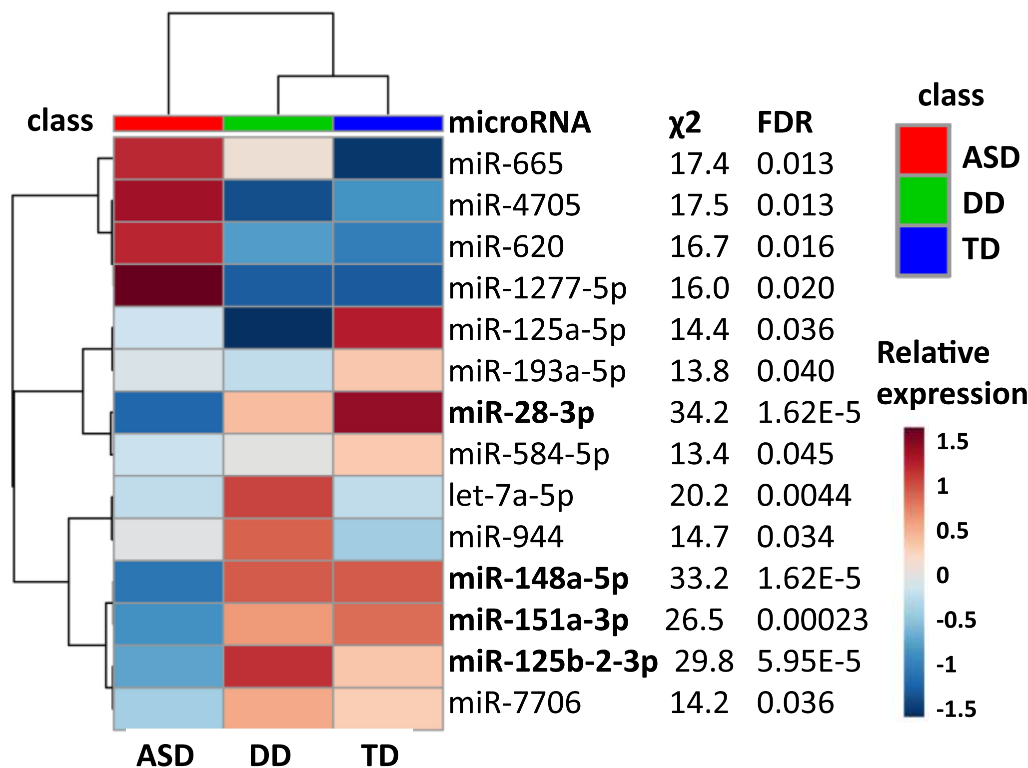
B. Test Set

Characteristic	All Groups (n = 62)	ASD (n = 37)	TD (n = 8)	DD (n = 25)
Demographics and anthropometrics				
Age (mo), mean (SD)	47 (14)	47 (14)	56 (14)	44 (14)
Boys, n (%)	49 (79)	29 (78)	5 (63)	15 (88)
Caucasian, n (%)	53 (85)	31 (84)	8 (100)*	14 (82)
BMI (kg/m^2), mean (SD)	17.5 (4)	16.9 (3)	19.9 (9)	17.6 (2)
Clinical characteristics				
Asthma, n (%)	4 (6)	4 (11)	0 (0)*	0 (0)*
GI disturbance, n (%)	12 (19)	6 (16)	1 (13)	5 (29)
ADHD, n (%)	6 (10)	6 (16)	0 (0)*	0 (0)*
Allergic rhinitis, n (%)	10 (16)	5 (14)	0 (0)*	5 (29)
Neuropsychiatric factors				
Communication by VABS-II standard score, mean (SD)	79 (23)	69 (21)	108 (13)*	79 (15)
Socialization by VABS-II standard score, mean (SD)	78 (26)	65 (20)	115 (9)*	79 (19)
Activities of daily living by VABS-II standard score, mean (SD)	81 (25)	69 (16)	113 (17)*	83 (24)
Social affect by ADOS-II score, mean (SD)	—	13 (5)	—	12 (6)
Restrictive/repetitive behavior by ADOS-II score, mean (SD)	—	4 (2)	—	2 (2)
ADOS-II total score, mean (SD)	—	17 (7)	—	14 (7)

Note: *Demographics, anthropometrics, clinical characteristics, and neuropsychiatric metrics are presented for the training set (A) and the test set (B). Clinical characteristics relevant to autism or oropharyngeal RNA content are displayed. Neuropsychiatric measures include the Vineland Adaptive Behavior Scales Second Edition (VABS-II) and the Autism Diagnostic Observation Schedule Second Edition (ADOS-II). ADOS-II scores are not included for children with typical development (TD) in whom such testing is not clinically indicated. However, mean ADOS-II and VABS-II scaled scores are provided for children with autism spectrum disorder (ASD) and peers with non-autism developmental delay (DD). ADOS-II total scores are presented rather than a composite score because most children were evaluated with the ADOS-II Toddler Module, in which a composite score is not generated. ADHD = attention-deficit/hyperactivity disorder; BMI = body mass index; GI = gastrointestinal.*

*$p < .05$.

FIGURE 1 Salivary MicroRNAs (miRNAs) Are Differentially Expressed Across Groups



Note: The 14 miRNAs with differential expression (false discovery rate [FDR] < 0.05) across autism spectrum disorder (ASD; red; n = 187), developmental delay (DD; green; n = 69), and typically developing (TD; blue; n = 125) groups at Kruskal-Wallis testing are shown, in addition to χ^2 statistics. Colored boxes represent relative group expression (measured by Pearson distance metric) and miRNAs are clustered in the heatmap using a complete clustering algorithm.

DD (social = 5 ± 3; p = 2.0E-11; restricted/repetitive behavior = 1 ± 1; p = 3.1E-9). This resulted in higher total ADOS-II scores for the ASD group (16 ± 6) compared with the DD group (6 ± 4; p = 1.9E-13).

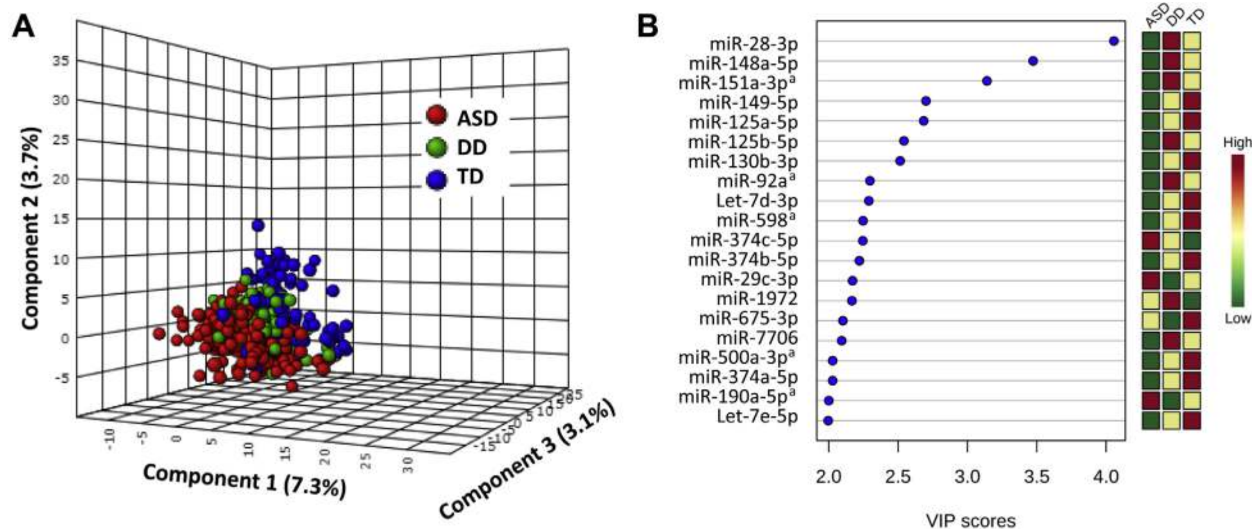
In the test set, children with ASD had lower standardized VABS-II communication scores (69 ± 21) than children with TD (108 ± 13), but not children with DD (79 ± 15). Children with ASD also displayed lower VABS-II socialization standard scores (65 ± 20) and activities of daily living scores (69 ± 16) than children with TD (115 ± 9 and 113 ± 7), but not children with DD (79 ± 19 and 83 ± 24). There was no statistical difference ($p > .05$) between the ASD and DD groups in ADOS-II measures.

We also examined potential differences in RNA quality metrics among sample groups. The ASD and non-ASD groups had mean RIN values of approximately 4.4 in our samples with no significant difference between the ASD and non-ASD groups (p = .7465 by unpaired t test) or among the 3 subgroups (F = 0058, p = .943 by analysis of variance). This also was consistent with a lack of difference in the RNA sequencing quality Q scores between the ASD and non-ASD groups (p = .0611 by t test) or among all 3 groups (F = 1.75, p = .173 by analysis of variance).

Expression of Salivary miRNA

Concentrations of 527 mature miRNAs were explored in the saliva of children with ASD, TD, and DD in the training set. Among the 527 miRNAs, 80 were present in the saliva of every participant. The miRNA with the highest salivary concentrations across all participants was miR-203a-3p, accounting for 1.14×10^6 of the total 8.44×10^7 raw read counts in the experiment (1.4%). Kruskal-Wallis nonparametric testing identified 14 miRNAs with significant (FDR < 0.05) differences across ASD, TD, and DD groups (Figure 1). The miRNA with the largest change was miR-28-3p (χ^2 = 34.2, FDR = 1.62E-5), which demonstrated downregulation in children with ASD relative to the TD and DD groups. Four other miRNAs demonstrated relative downregulation in the ASD group compared with the TD and DD groups (miR-148a-5p, miR-151a-3p, miR-125b-2-3p, and miR-7706). There were 4 miRNAs with relative upregulation in the ASD group compared with TD and DD groups (miR-665, miR-4705, miR-620, and

FIGURE 2 Salivary MicroRNA (miRNA) Profiles Separate Children With Autism Spectrum Disorder (ASD)



Note: *(A) Partial least squares discriminant analysis was used to map all 381 children in 3-dimensional space based on expression of the 527 salivary miRNAs. The analysis demonstrated nearly complete separation of children with ASD (red dots; n = 187) from children with typical development (TD; blue dots; n = 125) while accounting for 14.1% of the variance. There was incomplete spatial separation between children with ASD and children with non-autism developmental delay (DD; green dots; n = 69). (B) Variable importance in projection (VIP) scores were determined for the 527 individual miRNAs, and the 20 miRNAs with VIP score of at least 2.0 are shown. Color scales display relative projection importance across the ASD, TD, and DD groups.*
[a]Identified in previous miRNA studies involving human participants.

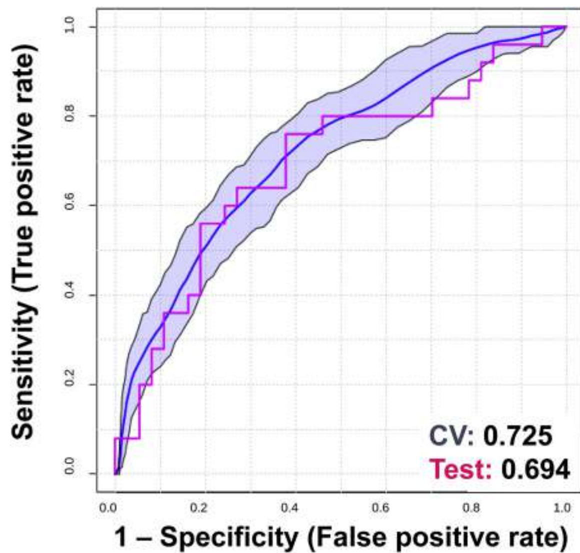
miR-1277-5p). One of the 14 miRNAs (miR-151a-3p) had been identified as "altered" in previous studies of miRNA expression in persons with ASD.[21] The remaining 6 of 14 miRNAs identified at Kruskal-Wallis testing displayed intermediate concentrations in the ASD group (relative to TD and DD groups) or had nearly overlapping expression patterns with the TD or DD group.

The utility of salivary miRNA profiles for identifying ASD status was explored in the training set with PLS-DA. Individual participants were mapped in 3-dimensional space using salivary miRNA profiles for the 527 miRNAs. This approach resulted in nearly complete separation of the ASD and TD groups, with intermediate alignment of the DD group (Figure 2A). It accounted for 14.1% of the variance in salivary miRNA expression among participants. Importance of individual miRNAs in participant PLSD-DA projection was determined by the weighted sum of absolute regression coefficients (variable importance in projection). Twenty miRNAs displayed significant variable projection importance (score $\geq$ 2.0; Figure 2B). Six of these 20 miRNAs overlapped with the 14 miRNAs identified at Kruskal-Wallis testing (miR-28-3p, miR-148a-5p, miR-7706, miR-151a-3p, miR-125a-5p, and miR-125b-2-3p). Five of these 20 miRNAs overlapped with those identified in previous miRNA studies in persons with ASD (miR-151a-3p, miR-92a-3p, miR-598-5p, miR-500a-3p, and miR-190a-5p).[15,16,21,28-30]

Classification Accuracy

Logistic regression analysis with a 100-fold cross-validation procedure was used to define an miRNA-based algorithm that differentiated the ASD group from the non-ASD group in the training set (n = 381). Only the 28 miRNAs identified at PLS-DA/Kruskal-Wallis analyses were interrogated, and medical/demographic variables were included as covariates. An algorithm using 4 miRNAs (miR-28-3p, miR-151-a-3p, miR-148a-5p, and miR-125b-2-3p), while controlling for sex, family ASD history, disordered sleep, GI disturbance, and presence/absence of chronic medical conditions, correctly identified 125 of 187 children with ASD and 129 of 194 children without ASD (Figure 3). This represented an AUC of 0.725 (95% CI 0.650–0.785). Notably, the 4 miRNAs included in this algorithm were identified by PLS-DA and Kruskal-Wallis analyses. Accuracy of the algorithm was prospectively assessed in the naïve test set (n = 62). The same algorithm identified 33 of 37 children with ASD and 8 of 25 children without ASD in the test set (AUC = 0.694). This represents a sensitivity of 89.2% and a specificity of 32.0%. Among children without ASD in the test set, the algorithm was more accurate at

FIGURE 3 Salivary MicroRNA (miRNA) Identify Autism Spectrum Disorder (ASD) Status



Note: A logistic regression analysis explored the ability of 28 miRNAs for identifying ASD status while controlling for medical/demographic covariates. A panel of 4 miRNAs (miR-28-3p, miR-148a-5p, miR-151a-3p, and miR-125b-2-3p) that controlled for sex, disordered sleep, attention-deficit/hyperactivity disorder (ADHD), family history (Hx) of ASD, gastrointestinal (GI) disturbance, and chronic medical conditions demonstrated an area under the curve (AUC) of 0.725 (95% CI 0.650–0.785) in the training set (n = 381) using a 100-fold cross-validation (CV) approach (blue line). This panel maintained an AUC of 0.694 in the naïve test set (n = 62), identifying 33 of 37 children with ASD and 8 of 25 peers without ASD. Equation: logit(P) = log(P/[1 − P]) = −0.085 + (10,199.182 × sleep disorder/miR-28-3p) + (0.014 × medication/miR-28-3p) + (10,199.207 × family Hx ASD/miR-151a-3p) + (0.042 × GI disturbance/miR-28-3p) − (10,199.229 × sleep disorder/miR-151a-3p) − (0.029 sleep disorder/miR-148a-5p) − (10,199.233 × family Hx ASD/miR-28-3p) − (0.045 × sleep disorder/miR-125b-2-3p) + (0.021 × ADHD/miR-28-3p) − (0.058 × sex/miR-28-3p) − (0.012 × pregnancy complications/miR-28-3p) − (0.024 × any medical condition/miR-28-3p).

differentiating those with TD (4 of 8) than those with DD (4 of 17).

Expression of Salivary miRNA Across ASD Phenotypes

Salivary miRNA expression patterns were explored across ASD phenotypes for children with ASD in the training set (n = 187; Table S1, available online). Significant correlations ($R > 0.25$, FDR < 0.05) were identified (Table 2) between salivary miRNA levels and presence of GI disturbance (2 miRNAs), but not ADHD. Among all salivary miRNAs, 5 miRNAs correlated with the standardized score on the socialization component of the VABS-II, 2 of which (miR-379-5p and miR-221-3p)[14,31,32] had been previously identified in ASD studies. There were no miRNAs correlated with communication or activities of daily living scores on VABS-II testing. Eight miRNAs were correlated with social affect on the ADOS-II. Six of these miRNAs were previously identified in ASD studies (miR-223-3p, miR-142-3p, miR-182-5p, miR-142-5p, miR-181c-5p, and miR-148b-3p),[17] and 1 displayed between-groups differences in the present study (miR-125b-2-3p) and was used in the logistic regression algorithm. Adjustment of these correlations based on RIN scores or RNA sequencing *Q* scores did not change them substantially and all remained highly significant (not shown). Ten miRNAs correlated with restricted/repetitive behavior on the ADOS-II, and 4 of these had been identified in previous ASD studies (miR-136-3p, miR-106a-5p, miR-130a-3p, and miR-431-5p).[17] Notably, all 10 were *positively* correlated with restricted/repetitive behavior score. Six miRNAs were correlated with total score on the ADOS-II, and all 6 had been identified in previous ASD miRNA studies.[17] As before, adjustment of these correlations based on RIN or *Q* scores did not change them substantially. All remained highly significant (not shown). One of these miRNAs (miR-151a-3p) was downregulated in children with ASD compared with children with TD and DD, and this miRNA was used in the logistic regression algorithm.

Influences of Clinical Characteristics on miRNA Expression

Associations of salivary miRNA expression and clinical/demographic characteristics were assessed in the training set (n = 381) with Pearson (continuous) or Spearman rank (dichotomous) correlation testing (Table S2, available online). There were no significant associations ($R < 0.25$, FDR < 0.05) between expression of the 527 miRNAs and participant sex, ethnicity, body mass index, dietary restrictions, asthma status, or allergic rhinitis status. Time of saliva collection had the largest number of miRNA associations compared with other medical/demographic variables tested (n = 21). The strongest association was between miR-210-3p levels and time of saliva collection ($R = -0.35$; $t = -6.6$; FDR = 4.2E-8). One miRNA (miR-23b-3p) was associated with time since last meal ($R = 0.25$; $t = 4.2$; FDR = 0.012). Of the 22 miRNAs associated with time of collection or time since last meal, 12 had been identified as potential biomarkers in previous miRNA studies.[17] One was "altered" in the saliva of children with ASD in the present study (miR-151a-3p; $R = -0.17$, FDR = 0.011). Given the importance of age in developing biomarker toolsets, it is worth noting that participant age was weakly ($R < 0.25$) yet significantly (FDR < 0.05) associated with 34 miRNAs. None of these miRNAs were used in the present biomarker panels, but 15 had been identified as potential targets in previous ASD miRNA studies.[17]

Functional Interrogation of miRNA Clusters

The mRNA targets and associated KEGG pathways for miRNA clusters of interest (ie, miRNAs identified at

TABLE 2 Salivary MicroRNAs (miRNA) Levels Associated With Autism Characteristics

Characteristics	miRNAs (*R*, FDR)
GI disturbance	miR-4700-3p[a] (0.37, 6.33E-05); miR-4485-3p (−0.27, 0.043)
ADHD	
VABS commun	
VABS social	miR-152-3p (0.30, 0.023); miR-379-5p[a] (−0.30, 0.023); miR-4781-3p (−0.28, 0.038); miR-26a-5p (−0.28, 0.039); miR-221-3p[a] (0.28, 0.039)
VABS ADLs	
ADOS social	miR-223-3p[a] (0.33, 0.0081); miR-142-3p[a] (0.33, 0.0082); miR-182-5p[a] (−0.32, 0.016); miR-142-5p[a] (0.31, 0.016); miR-125b-2-3p[b] (−0.29, 0.035); miR-181c-5p[a] (0.29, 0.036); miR-148b-3p (−0.29, 0.036); miR-143-3p[a] (0.28, 0.044)
ADOS RRB	miR-136-3p[a] (0.52, 1.70E-08); miR-8485 (0.42, 3.21E-05); miR-106a-5p[a] (0.38, 0.00051); miR-3679-5p (0.36, 0.0010); miR-573 (0.33, 0.0049); miR-6733-5p (0.30, 0.021); miR-8061 (0.29, 0.025); miR-130a-3p[a] (0.28, 0.040); miR-766-5p (0.28, 0.045); miR-431-5p[a] (0.28, 0.045)
ADOS total	miR-223-3p[a] (0.34, 0.0043); miR-142-3p[a] (0.34, 0.0044); miR-142-5p[a] (0.31, 0.015); miR-182-5p[a] (−0.31, 0.015); miR-148b-3p (−0.30, 0.020); miR-151a-3p[a,b] (−0.28, 0.049)

Note: The miRNAs significantly associated (false discovery rate [FDR] < 0.05) with autistic features among 187 children with autism spectrum disorder (ASD; training set) are shown. Pearson R values and FDR-corrected p values are displayed. ADHD = attention-deficit/hyperactivity disorder; ADLs = activities of daily living; ADOS = Autism Diagnostic Observation Schedule; commun = communication; GI = gastrointestinal; RRB = restrictive repetitive behavior; VABS = Vineland Adaptive Behavior Scales.

[a]MiRNAs identified in previous human studies of autism.

[b]MiRNAs with between-groups differences in present study.

Kruskal-Wallis testing or associated with ASD features on ADOS-II) were explored in DIANA miRPATH software. The 14 miRNAs "altered" among the ASD, TD, and DD groups had a total of 9,169 mRNA targets (microT-cds score $\geq$ 0.8, $p < .05$), 5,997 of which were unique (Table S3A, available online). MiR-1277-5p accounted for the largest number of mRNA targets (n = 2,914; 31.8%). The mRNA targets over-represented (FDR < 0.05) 41 KEGG pathways (Table S4A, available online). Brain-related KEGG pathway targets included prion diseases (FDR = 1.8E-6; 10 mRNAs, 5 miRNAs); morphine addiction (FDR = 2.2E-6; 41 mRNAs, 11 miRNAs), phosphoinositide 3-kinase–Akt signaling (FDR = 3.8E-5; 154 mRNAs, 13 miRNAs), axon guidance (FDR = 4.1E-4; 63 mRNAs, 11 miRNAs), Wnt signaling (FDR = 0.0029; 64 mRNAs, 12 miRNAs), γ-aminobutyric acid–associated (GABAergic) synapse (FDR = 0.0043; 35 mRNAs, 10 miRNAs), glioma (FDR = 0.007; 31 mRNAs, 10 miRNAs), retrograde endocannabinoid signaling (FDR = 0.019; 45 mRNAs, 12 miRNAs), and circadian entrainment (FDR = 0.029; 42 mRNAs, 12 miRNAs). Hierarchical clustering of the 14 miRNAs based on KEGG pathway union yielded 5 distinct groups of miRNAs (Figure S1, available online). Remarkably, 3 pairs of miRNAs (miR-193a-5p/miR-125a-5p; miR-148a-5p/miR-944; and miR-620/miR-4705) demonstrated functional clustering patterns that mirrored hierarchical clustering based on their salivary expression levels (Figure 1). Analysis of the 231 most high-confidence mRNA targets (experimentally validated miRNA-mRNA interaction, microT-cds $\geq$ 0.975) in String software showed greater functional connections of mRNA protein products than expected by chance alone (protein-protein interaction enrichment p = 1.1E-8). The 231 protein products had 270 functional connections and a clustering coefficient of 0.35. The 14 miRNAs also targeted 436 of the 961 autism candidate genes in the SFARI gene database, exceeding the 288 targets expected by chance alone ($\chi^2 = 54.7$, $p < .0001$).

Analysis of the 8 miRNAs associated with ADOS-II total/socialization scores also showed brain-related mRNA target pathways. The 8 miRNAs had a total of 4,147 mRNA targets, 3,311 of which were unique (Table S3B, available online). There were 2 miRNAs (miR-182-5p and miR-142-5p) that accounted for 2,064 (49.8%) of total mRNA targets. The mRNA targets over-represented 47 KEGG pathways (Table S4B, available online). Brain-related KEGG pathway targets included prion disease (FDR = 2.1E-13; 9 mRNAs, 6 miRNAs), long-term depression (FDR = 0.0017; 23 mRNAs, 7 miRNAs), morphine addiction (FDR = 0.0017;

26 mRNAs, 7 miRNAs), phosphoinositide 3-kinase–Akt signaling (FDR = 0.0017; 93 mRNAs, 8 miRNAs), glioma (FDR = 0.0072; 21 mRNAs, 8 miRNAs), retrograde endocannabinoid signaling (FDR = 0.0085; 34 mRNAs, 7 miRNAs), nicotine addiction (FDR = 0.0116; 13 mRNAs, 5 miRNAs), neurotrophin signaling (FDR = 0.0134; 38 mRNAs, 8 miRNAs), glutamatergic synapse (FDR = 0.0180; 36 mRNAs, 7 miRNAs), oxytocin signaling pathway (FDR = 0.0207; 43 mRNAs, 7 miRNAs), cholinergic synapse (FDR = 0.0207; 37 mRNAs, 8 miRNAs), GABAergic synapse (FDR = 0.0238; 23 mRNAs, 7 miRNAs), and axon guidance (FDR = 0.0267; 34 mRNAs, 7 miRNAs). Analysis of the 203 most high-confidence mRNA targets in String software showed greater connectedness than that expected by chance alone (protein-protein interaction enrichment p = 1.1E-5). There were 215 node connections among the 203 protein products, with a clustering coefficient of 0.30. The 8 miRNAs also targeted 237 of the 961 SFARI autism candidate genes, exceeding the 159 gene targets expected by chance alone ($\chi^2 = 31.8$, $p < .0001$).

DISCUSSION

This prospective case-control study of 443 children (2–6 years old) identified 28 salivary miRNAs with varying levels in children with ASD, TD, or DD. A panel using 4 miRNAs distinguished ASD status in the training and naïve test sets. A subset of salivary miRNAs was associated with measures of adaptive and ASD behaviors. Together, these groups of miRNAs targeted genes strongly related to neurodevelopment and implicated in ASD pathogenesis (Table S5, available online).

There are a number of potential environmental factors that can disrupt levels of miRNAs in the oropharynx of children with ASD. Certainly, dietary restrictions in children with ASD[18] can alter the salivary miRNA milieu. However, the present study found no associations between saliva miRNA levels and the presence of dietary restrictions, and only 2 miRNAs were strongly associated with GI disturbance. In addition, there was no difference in the rate of dietary restrictions among the ASD, DD, and TD groups. A second potential mechanism for salivary miRNA disruption could be differences in dental hygiene, given the resistance of many children with ASD to brushing their teeth.[33] For this reason, this study specifically excluded children with active dental infections or decay. There are alterations in the oral microbiome of children with ASD[34] that can drive a portion of salivary miRNA changes, but oral microbiome differences in children with ASD are largely unrelated to the bacteria implicated in dental carries.[35]

Children with ASD experience difficulties with oral-motor (speech apraxia) and oral-sensory (food texture sensitivity) processing.[19,36] The cranial nerves that guide these processes could contribute to salivary miRNA patterns. Brain relatedness of the salivary miRNAs identified in this study is supported by the functions of their mRNA targets, which include axonal guidance, neurotrophic signaling, GABAergic synapse, and addiction pathways (Tables S4A and S4B). For example, miR-148a-5p (used in the diagnostic panel of the present study) targets 7 mRNAs involved in axon guidance (Table S3A, available online), and 2 of these (SLIT3 and SRGAP3) are autism candidate genes.[27] The SLIT3 protein product acts as a molecular guidance cue in axonal outgrowth by interacting with the protein product of another autism candidate gene, ROBO1.[37] Notably, ROBO1 is a target of miR-944 (Table S3A, available online), an miRNA associated with ASD status in the present study, and highly correlated with miR-148a-5p in concentration (Figure 1) and function (Figure S1, available online). The parallel functions of miR-944 and miR-148a-5p in axon guidance, coupled with their overlapping expression in children with ASD, highlight their potential significance in ASD pathophysiology.

The glymphatic system represents yet another potential route for salivary entry of brain-related miRNAs. The anatomic proximity of the perivascular drainage spaces in the glymphatic system to the oropharynx creates a prospective avenue for gut-brain cross-talk and miRNA transfer.[11] In light of the pronounced diurnal activity displayed by the glymphatic system,[38] indirect support for this transfer might lie in the surprising correlations between salivary miRNA levels and time of collection (Table S2, available online). In addition, the mRNA targets of ASD-associated miRNAs show enrichment for circadian-related pathways (Table S4A, available online), which is notable because disordered sleep is a common medical condition in children with ASD.[39]

The potential relevance of salivary miRNA levels to ASD behavior is underscored by the large number of salivary miRNAs associated with measures of ASD symptoms on the ADOS-II (Table S1, available online). Previous studies have described miRNAs as "altered" in persons with ASD relative to healthy control participants.[17] The increased power of the present investigation provides an opportunity to explore miRNA patterns among ASD phenotypes. We identified 8 miRNAs associated with social affect and 10 miRNAs associated with restricted/repetitive behavior. Such associations might be driven by robust miRNA "alterations" in a subset of children with a similar single-nucleotide polymorphism or copy number variant.[40] In these children, phenotypic similarities might result from genetic mutations that produce a direct miRNA change or lead to compensatory miRNA

responses. One example is miR-106a-5p.[41] This miRNA has been previously identified in 3 separate ASD studies of postmortem brain,[30] blood,[21] and lymphoblasts.[15] It targets 20 mRNAs involved in axon guidance (Figure S1, available online),[25] including 4 autism candidate genes (SEMA5A, NTNG1, SRGAP3, and MAPK1).[27] We found that miR-106a-5p levels were directly associated with restricted/repetitive behavior in children with ASD (Table 2). Thus, altered levels of miR-106a-5p could target key transcripts involved in brain development that underlie restricted/repetitive behaviors. Additional studies tracking expression patterns of such miRNAs and behavioral therapy interventions are warranted before strong conclusions can be drawn.

This study defines an algorithm using 4 miRNAs to differentiate children with ASD from peers with TD or DD (Figure 3). In a naïve test set, the panel demonstrated 89% sensitivity and 32% specificity. This accuracy approaches that of subjective measures currently used (eg, MCHAT-R[7]), with the added benefit of being fast, objective, and noninvasive. Emerging biomarker work in eye tracking,[3,42] imaging,[43] genetic,[44] and electrophysiologic markers[45] also has shown considerable promise for identifying ASD status. The future of ASD evaluation will likely involve a multifactorial approach using each of these components in concert. The results of this study suggest that salivary RNA biomarkers deserve strong consideration in this field. Indeed, bolstering the present algorithm with a poly-"omic" analysis of additional RNA families has led to an even more comprehensive and accurate approach.[46]

Among the 4 miRNAs used in the diagnostic algorithm, 2 (miR-125b-2-3p and miR-151a-3p) were strongly associated with ASD traits at ADOS evaluation (Table 2) and 1 (miR-151a-3p) was identified in previous studies.[17] Limited overlap with previous miRNA studies might have resulted because blood and lymphoblast miRNAs are not reliably transferred to (or expressed in) saliva. This finding also might reflect limited generalizability of small cohort studies to a large heterogeneous population of children with ASD. Levels of certain miRNAs can vary widely from child to child depending on many factors (eg, time of collection, comorbid medical conditions, age, and sex). For this reason, "outlying" miRNA concentrations in just a few individuals could lead to the assumption that between-group differences exist, when the mean group expression is effectively biased by just a few samples. Small studies (ie, nearly all previous studies of miRNAs in persons with ASD) are particularly prone to this. In the present study, we used a large sample and complementary Kruskal-Wallis and PLSDA approaches to select miRNAs, which avoid this pitfall.

It also is notable that many previously identified miRNA biomarkers (11 miRNAs) demonstrated associations with time of collection (Table S2, available online). This factor has not been routinely considered in previous ASD miRNA studies. Given recent findings that a significant proportion of serum-based miRNAs demonstrate diurnal variation,[47] these findings also likely apply to blood-based biomarkers. Further studies examining the interaction between miRNA expression and circadian rhythm could be important in understanding the role of these molecules in sleep-wake cycles and provide valuable information in the development of miRNA biomarkers for clinical application. Importantly, there were no differences in collection time among the ASD, TD, and DD groups in this study.

Surprisingly, there was little overlap between the salivary miRNAs identified in our pilot investigation and those identified in the present study.[20] This might have resulted from 3 important differences in study protocols. First, the pilot study used expectorated saliva, whereas the present investigation collected saliva with a swab technique. This change was made because children with ASD have difficulty producing expectorant on command. It might have led to differences in ratios of cell-derived and (vesicle) carrier-derived miRNA. Second, the pilot study involved children 5 to 14 years of age, whereas the present study enrolled children 2 to 6 years of age. This change was made to capture children at the age when ASD diagnosis is first made and screening/diagnostic testing is most needed.[1] It might have influenced a subset of miRNAs with age-related expression. Third, the pilot study targeted children with "high functioning" ASD (average ADOS-II score $= 10.6 \pm 4.1$), whereas the present large follow-up study included all children with ASD regardless of severity (average ADOS-II score $= 16 \pm 6$). Because salivary miRNA expression is associated with levels of ASD symptoms (measured by ADOS-II), it is likely that expanding the present study to include a heterogeneous population of children with ASD led to changes in observable between-group differences.

There are numerous medical and demographic factors that must be considered when identifying and testing physiologic biomarkers. The prospective nature of the present study allowed us to control for many of these factors by using identical collection, storage, and sample processing techniques across groups. We also attempted to match groups based on relevant factors such as age, sex, ethnicity, body mass index, and time of collection. Unfortunately, complete matching of all factors is nearly impossible. As a result, the training set displays between-group differences in age and sex. However, it is worth noting that the age range used in the present study (2–6 years) is extremely tight compared with many biomarker studies and the resulting age difference between ASD and TD groups (7 months) is unlikely to have significant bearing on miRNA expression. In

addition, none of the miRNA biomarkers identified in this study demonstrated significant correlations with age or sex and the multivariate regression algorithm controls for sex.

Another extremely important topic to consider when assessing the veracity of RNA research is nucleic acid integrity and its potential influence on biomarker outcomes. Although we report RIN values across the 3 groups of samples, it is important to note that this metric likely underestimates RNA quality in miRNA-enriched samples. Unlike longer messenger RNAs, small RNAs (eg, miRNA, piwi-interacting RNA, and small nucleolar RNA) are relatively resistant to salivary endonucleases. As a result, even samples with low RIN values (and presumably poor RNA quality) can demonstrate excellent miRNA yields on bio-analyzer output (Figure S2, available online). Indeed, a study using human cell and tissue samples subjected to total RNA purification after longitudinal heat degradation has demonstrated that RIN values rapidly decrease with heat exposure and housekeeping messenger RNAs are lost to detection, whereas miRNAs remain remarkably stable over time.[48] Despite the limits associated with RIN reporting, we note that the average RIN for this dataset exceeded RIN values reported in previous saliva RNA studies[49]; there was no difference in average RIN among the ASD, TD, and DD groups; and RNA-ADOS correlations were actually strengthened when RIN was added as a covariate. We encourage any future studies using saliva RNA measures to use stringent methods for RNA stabilization and extraction and to carefully assess the influence of RNA integrity on biomarker findings.

This study provides large-scale evidence that salivary miRNA can be used to differentiate children with ASD from peers with TD or non-autism DD. It shows that levels of salivary miRNAs are correlated with measures of adaptive and ASD behaviors, and that these miRNAs target pathways that are implicated in ASD pathogenesis. Improving specificity of the defined salivary miRNA algorithm is crucial for clinical utility. This has been achieved through a multimodal approach using additional "-omic" measures.[46] Additional characterization of the factors that influence salivary miRNA expression also will be crucial.

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