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Abstract

Autism Spectrum Disorder (ASD) is a complex human neurodevelopmental condition with increasing prevalence, yet early diagnosis remains challenging, particularly in low- and middle-income countries such as Brazil, where molecular studies are scarce. This study investigated the salivary expression of four microRNAs (miR-23a-3p, miR-140-3p, miR-151a-3p, and miR-486-3p) in 45 Brazilian adults (24 with ASD and 21 neurotypical controls), using RT-qPCR and bioinformatic analyses of functional enrichment and gene target prediction. The results demonstrated significant overexpression of miR-23a-3p (FC=28.8-fold; p=0.0012), miR-140-3p (p=0.0352), and miR-486-3p (p=0.0352) in the ASD group, as well as co-expression among these miRNAs. miR-23a-3p showed the best diagnostic accuracy (AUC=0.827; specificity=100%), while miR-486-3p exhibited differential expression associated with the year of diagnosis (p=0.012). A very strong correlation was observed between miR-23a-3p and miR-151a-3p expression (ρ=0.973), along with co-regulation of gene targets across salivary gland, prefrontal cortex, and hypothalamic tissues. Enrichment analyses revealed association of gene targets with processes central to neurodevelopment, including forebrain and telencephalon development, cognition, social behavior, synaptic plasticity, and synaptic vesicle cycling. These findings provide novel evidence of salivary microRNA dysregulation in Brazilian adults with ASD, a demographic notably underrepresented in molecular autism research. Collectively, the evidence indicates that specific microRNAs involved in synaptic regulation are dysregulated in ASD and highlights the potential of saliva as a non-invasive and accessible biofluid for molecular screening in neurodevelopmental disorders.

Keywords: microRNA; ASD biomarkers; salivary diagnostics; Brazilian population; Autism;

1. Introduction

Autism Spectrum Disorder (ASD) is a major global public health concern, with an estimated 61.8 million individuals affected worldwide in 2021 [1]. In Brazil, however, ASD prevalence remains uncertain due to limited nationwide epidemiological data and a lack of standardized, validated diagnostic tools [2, 3]. As a low and middle-income country (LMIC), Brazil's vast territory poses significant challenges for conducting large-scale epidemiological studies across diverse populations [4]. In an effort to address this gap, the Brazilian federal government included ASD-related data collection in the 2020 national census, however, the results are still under investigation [5]. More generally, if the estimated ASD prevalence of 1% is applied to Brazil's population, it can be projected that approximately 2 million individuals are affected [5]. These factors highlight the need for comprehensive and regionally representative studies to better understand the distribution of ASD across diverse populations in Brazil.

Despite significant advances, the underlying biology of ASD remains particularly complex, shaped by intricate interactions between genetic and epigenetic mechanisms in response to diverse environmental influences [6]. In this regard, a group of small non-coding RNAs called microRNAs has received progressive attention, suggesting that they can be used as a diagnostic tool in the form of a biomarker [7].

It is widely accepted that altered miRNA profiles in ASD were first documented in postmortem cerebellar tissue [8]. Subsequent investigations have demonstrated widespread dysregulation across the central nervous system (CNS) and peripheral compartments, including lymphoblasts, blood, saliva, and olfactory precursor cells [9-12].

Among the epigenetic factors contributing to ASD, in recent years, intense research has focused on demonstrating that specific salivary microRNAs are highly accurate in differentiating children with ASD from children with typical development [13], providing evidence that this technology can be launched commercially for use in clinical practice as a diagnostic purpose.

Salivary microRNA, in particular, have shown promise as noninvasive biomarkers for distinguishing individuals with ASD from typically developing controls [14]. However, studies exploring microRNA profiles in Brazilian populations are lacking.

Given the lack of information regarding the roles of salivary microRNAs roles in ASD and their potential as reliable biomarkers within the Brazilian population, this study investigates the salivary expression profiles of four circulating microRNA (miR-23a-3p, miR-140-3p, miR-151a-3p, and miR-486) in adults with and without ASD from Brazil. The findings aim to contribute to the development of regionally informed molecular tools to support early ASD identification complementing standard developmental screening tests to improve early detection in this population.

2. Materials and Methods

Participant characterization

The study design was characterized by two comparative neurodivergent groups, including 45 adult volunteer participants. The inclusion criterion for participation in this study consisted of a minimum age of 18 years, and written consent from the participant or legal guardian was obtained from all volunteers. The study was approved by the Institutional Ethics Committee of Universidade Católica Dom Bosco (CAAE No. 56241522.9.0000.5162).

The ASD group consisted of 24 adult samples (F=11; M=13), where clinical screening for the ASD group involved the inclusion of patients with a clinical diagnosis of autism, according to the parameters of the Diagnostic and Statistical Manual of Mental Disorders (DSM-5) [15]. In comparison, the control group consisted of 21 adults (F=4; M=17) with neurotypical development, with the presence of reported neurodevelopmental disorders being an exclusion criterion. Additional participant information, including year of diagnosis, support level, clinical comorbidities, parental age, and number of siblings, is summarized in Table 1. Individuals with previously documented genetic mutations were excluded from the study. Both groups reported no history of upper respiratory tract infections during the week prior to saliva collection.

Salivary sample collection and storage

Saliva was chosen as the primary biofluid for this study due to its non-invasive collection protocol compared to blood collection methodologies. This approach minimizes participant discomfort and stress, which is a crucial ethical and practical consideration for research involving individuals with autism spectrum disorder, thereby enhancing recruitment

feasibility and protocol adherence. Saliva samples from both groups were obtained using sterile 15-cm swabs without culture substrate (ABSORVE CRALPLAST™), collected after one hour of absolute fasting during the morning period, with water consumption permitted to stimulate salivation [16]. The collection procedure involved absorbing saliva from the oral cavity by applying rotational force with the swab onto the epithelial surfaces of the tongue, cheeks (right and left), and the sublingual region, totaling one minute per collection. The swab was then returned to its respective storage tube, and 2 mL of RNAlater™ (Sigma-Aldrich) was added to completely immerse the absorption tip, ensuring the preservation of total RNAs from the biological samples. Immediately after collection, samples were sealed with parafilm, labeled, and kept on ice for transport to the laboratory, where salivary microRNA extraction was performed. All saliva samples from participants in this study were collected in biological duplicates.

Salivary microRNA extraction

Samples underwent microRNA extraction using the miRNeasy Mini kit (Qiagen) with adaptations for salivary microRNA extraction from swabs. In the initial step, the collected swab was transferred to a sterile 2-mL microtube using sterilized forceps cleaned with 0.5% diethylpyrocarbonate (DEPC). The absorption tip was then cut from the swab shaft using sterilized scissors under previously described conditions. In this new microtube, 1 mL of T.E. buffer (0.01 M Tris-HCl, 0.001 M EDTA, RNase-free water, pH 8.0) was added to precipitate the biological material. The sample was vortexed for 1 minute to homogenize and facilitate swab removal. Subsequently, the buffered solution containing the biological pellet was centrifuged at 10.000 rpm for 2 minutes at 4°C, and the supernatant was discarded at the end of the process. After obtaining the biological pellet, the material followed the manufacturer's instructions for continuation of microRNA extraction. At the start of extraction, for quality control monitoring, 1 µL of synthetic UniSpike-in 6 RNA template (10^8 copies/µL) was added to each sample prior to the addition of QIAzol Lysis Reagent. In this study, amplification of the UniSpike-in 6 unit was also used as an exogenous reaction control to normalize salivary microRNA amplification quality by RT-qPCR. Upon completion of microRNA extraction, samples with a qualitative profile of 1.8–2.0 (A260/280 absorbance) and >10.0 ng/µL were selected for RT-qPCR validation.

Reverse Transcription (RT) and Real-Time PCR (qPCR)

Quantitative assessment and relative expression of neuroclinical biomarkers were performed using a set of microRNA primers. Following the findings reported by [17], four circulating miRNAs (hsa-miR-23a-3p, hsa-miR-140-3p, hsa-miR-151a-3p, and hsa-miR-486-3p) were selected for targeted quantification and analysis as candidate indicators of ASD prognosis. The microRNAs selected for this study were analyzed using LNA technology primers from the miRCURY LNA miRNA PCR Starter Kit (Qiagen), following the manufacturer's instructions. cDNA samples were diluted in RNase-free water at a 1:30 ratio and applied to 96-well plates for qPCR analysis. Each reaction was performed in technical triplicates with a final reaction volume of 10 μL per well. RT-qPCR was conducted using the QuantStudio™ 3 Real-Time PCR System according to the manufacturer's protocol.

Statistical analyses

Sample quality control was performed based solely on technical triplicates with a coefficient of variation (CV) below 10%. Data normalization was performed using the ΔCt method, with miR-103a-3p as the endogenous control, and relative expression was calculated as log2(Fold Change) using the $2^{-\Delta\Delta Ct}$ formula. Data normality was assessed by the Shapiro-Wilk test, and homogeneity of variances was assessed by Levene's test. For between-group comparisons, the Wilcoxon–Mann–Whitney test with False Discovery Rate (FDR) correction was applied, and the effect size of the magnitude of difference was estimated using Cliff's Delta. Diagnostic accuracy was evaluated using ROC curve analysis, with calculation of the area under the curve (AUC), 95% confidence interval, and curve comparison performed using DeLong's test. Correlations were analyzed using Spearman's correlation coefficient. Complementary analyses were performed by ASD subcategories, including gender, support level, and DSM category. All analyses were performed in the RStudio statistical environment (2026.05.0+218), with the aid of the packages 'rstatix' for statistical tests, 'pROC' for ROC curves, 'effsize' for Cliff's Delta, 'car' for Levene's test, as well as 'ggplot2', 'dplyr', and 'tidyr' for data manipulation and visualization. All analyses were performed with statistical significance set at $p < 0.05$.

Enrichment analyses

For the identification of gene targets regulated by the microRNAs of interest, target transcript identification of the selected microRNAs was performed using the TargetScanHuman 8.0 prediction tool [18]. Selected target transcripts were obtained from predictions for hsa-miR-23a-3p, hsa-miR-151a-3p, hsa-miR-486-3p, and hsa-miR-140-3p (isoforms .1 and .2). Cumulative Weighted Context++ Score (CWCS) values were used as the evaluation criterion, with more negative scores representing higher-confidence miRNA-target interactions. Only interactions with human genes were selected. In parallel, tissue expression data (nTPM - transcripts per million) were obtained from the Human Protein Atlas [19] using Genotype-Tissue Expression (GTEx) files for salivary gland, cerebral cortex, and hypothalamic tissues. The dataset was additionally compiled with data from the SFARI Gene database (Simons Foundation Autism Research Initiative) for genes reported to be associated with autism spectrum disorder. To reduce dimensionality and focus on the most robust interactions, genes with better CWCS interaction scores were selected, whereupon functional enrichment analyses were conducted using the clusterProfiler platform, focusing on Biological Processes by Gene Ontology (GO - BP). Statistical analyses were performed in the R environment (2026.05.0+218), using the dplyr, tidyr, ggplot2, ComplexHeatmap, circlize, and clusterProfiler packages. Overrepresented GO terms among the target genes of each microRNA were evaluated, considering terms with a Benjamini-Hochberg adjusted p-value < 0.05 as significant. For integrated data visualization, heatmap-type graphs were employed to represent tissue expression patterns and prediction scores and Pearson correlation. Finally, the ShinyGO 0.85.1 program was used for enrichment analysis via hierarchical clustering tree for cellular components and the Kyoto Encyclopedia of Genes and Genomes (KEGG), considering parameters of fold enrichment with < 0.05 FDR q-value (-log10) [20].

3. Results

Cohort Demographics and Clinical Profile

The demographic analysis indicated a median participant age of 23 years (IQR: 20–28), with no significant age difference between the control group (22 years; IQR: 20–24) and the ASD group (25 years; IQR: 21–28; $p = 0.4$) (Table 1). Sex distribution demonstrated a predominance of males in both groups (66.7%), with no significant difference between them ($p > 0.7$). Among individuals with a confirmed ASD diagnosis, the median age at diagnosis was 15 years (IQR: 5–19), and most were classified as support level 1 (79.2%), followed by

support level 2 (20.8%). Parental ages from ASD group exhibited substantial variability, ranging from 58 years for fathers (IQR: 48–63) and 57 years for mothers (IQR: 48–60), without a defined distribution pattern. Sibling distribution showed that 33.3% of participants were only children, 50% had up to two siblings, and 16.7% had three or more siblings.

Regarding early clinical suspicion, 45.8% of participants had no prior indication of ASD, whereas 33.3% reported an initial suspicion of the condition. Comorbid diagnoses were identified in 76.16% of individuals with ASD, including Atypical autism (15.7%), Schizophrenia (15.7%), Language impairment (15.7%), Generalized anxiety disorder (15.7%), Cognitive rigidity (10.5%), Intellectual disability (10.5%), Attention deficit hyperactivity disorder (5.2%), Depression (5.2%) and Epilepsy (5.2%).

Table 1. Demographic analysis.

Participant Characteristics	Overall N = 45[1]	Control N = 21[1]	ASD N = 24[1]	*p*-value[2]
Age	23 [20-28]	22 [20-24]	25 [21-28]	0.4
Gender				
F	15 (33.3%)	4 (19.0%)	11 (45.8%)	
M	30 (66.7%)	17 (81.0%)	13 (54.2%)	0.068
ASD support level				
1			19 (79.2%)	
2			5 (20.8%)	> 0.9
Age at definitive diagnosis			12 [5-19]	
Primary suspicion				
No			11 (45.8%)	
Yes			8 (33.3%)	> 0.9
Paternal age			58 [48-63]	0.7
Maternal age			57 [48-60]	
Number of siblings				
Only			8 (33.3%)	
Up 2			12 (50.0%)	0.5
> 3 siblings			4 (16.7%)	
Addictional diagnosis			Atypical autism (15.7%), Schizophrenia (15.7%), Language impairment (15.7%), Generalized anxiety disorder (15.7%), Cognitive rigidity (10.5%), Intellectual disability (10.5%), Attention deficit hyperactivity disorder (5.2%), Depression (5.2%), Epilepsy (5.2%).	0.9

[1]Continuous variables: Median [Q1, Q3]; Categorical variables: n (%)
[2]Wilcoxon rank sum test; Fisher's exact test;

Differential Expression Analysis

Normality testing of the data using the Shapiro-Wilk test showed that expression values for all four analyzed miRNAs (hsa-miR-23a-3p, hsa-miR-140-3p, hsa-miR-151a-3p, and hsa-miR-486-3p), transformed to log2FC, exhibited a normal distribution in both the control and ASD groups (Supplementary Table 1). In contrast, demographic variables, including age in both groups and the year of definitive diagnosis for the ASD group, violated the normality assumption, justifying the use of non-parametric tests for these variables. Levene's test for homogeneity of variances revealed significant heteroscedasticity between groups for miRNAs hsa-miR-23a-3p ($p=0.002$), hsa-miR-140-3p ($p<0.001$), and hsa-miR-151a-3p ($p<0.001$), indicating unequal variances between samples. Only hsa-miR-486-3p showed homogeneity of variances ($p=0.069$). Finally, Cliff's Delta analysis revealed that hsa-miR-23a-3p exhibited the largest effect size ($\Delta = 0.654$), classified as "large" according to conventional criteria (95% CI: 0.348–0.834). hsa-miR-140-3p ($\Delta = 0.425$) and hsa-miR-486-3p ($\Delta = 0.414$) presented "moderate" effect sizes, while hsa-miR-151a-3p demonstrated a "small" effect ($\Delta = 0.303$).

Relative Expression of Salivary microRNAs

The relative expression of the analyzed salivary microRNAs demonstrates a heterogeneous distribution among individuals in the ASD group (Figure 1). This intragroup variability was statistically pronounced for hsa-miR-23a-3p, hsa-miR-140-3p, and hsa-miR-486-3p, which exhibited differential expression levels compared to the Control group. In contrast, hsa-miR-151a-3p did not present a significant expression profile. Analysis using the Wilcoxon–Mann–Whitney test with FDR correction demonstrated that hsa-miR-23a-3p showed the highest level of statistical significance (adjusted $p = 0.0012$), with a median expression increase from 0.102 in the control group to 2.930 in the ASD group, corresponding to a fold change (FC) of 28.8-fold (log2FC = 4.85) (Table 2). hsa-miR-140-3p and hsa-miR-486-3p also showed significant differences after correction (adjusted $p = 0.0352$ for both), with medians in the ASD group of 2.910 and 2.450, respectively. hsa-miR-151a-3p, although showing an increasing trend (ASD median = 2.487 vs. control = 0.079), did not reach statistical significance after correction (adjusted $p = 0.094$).

Table 2. Comparative analysis of salivary microRNA expression between Control and ASD groups. Median values of relative log2(Fold Change) expression (log2FC) for microRNAs hsa-miR-23a-3p, hsa-miR-140-3p, hsa-miR-486-3p, and hsa-miR-151a-3p. Wilcoxon–Mann–Whitney test with False Discovery Rate (FDR) correction, adjusted $p < 0.05$.

miRNA	Mediana_control	Mediana_ASD	FC_ratio	log2FC	*p* valor	adjusted *p*
miR-23a-3p	0.102	2.930	28.844	4.850	<0.001	**
miR-140-3p	-0.266	2.910	-10.920		0.035	*
miR-151a-3p	0.079	2.487	31.438	4.974	0.094	ns
miR-486-3p	-0.313	2.450	-7.819		0.035	*

Complementary categorical data

The comparative t-test analysis regarding the year of definitive diagnosis of patients in the ASD group was categorized according to diagnostic criteria between the years of DSM-IV-TR updates (1995-2013) and DSM-5 (post-2013). Accordingly, the results reveal significant differences in miR-486-3p expression ($p = 0.012$) between the subcategories. Individuals diagnosed between 1995 and 2013 presented a median expression of 3.213 for miR-486-3p, while those diagnosed after 2013 demonstrated a median expression of -0.693, indicating a substantial reduction in the levels of this miRNA in samples diagnosed in more recent years (Supplementary Table 1). Although they did not reach statistical significance, miRNAs miR-23a-3p ($p = 0.084$), miR-140-3p ($p = 0.194$), and miR-151a-3p ($p = 0.098$) showed trends toward reduced expression in the group diagnosed after 2013, with median values of 0.442, 0.157, and -1.408, respectively, compared to the values of 2.930, 2.910, and 2.687 observed in the group of individuals diagnosed between 1995-2013. The comparison between support level 1 and support level 2 revealed no statistically significant differences in miRNA expression, nor were significant differences observed in miRNA expression between females and males in the ASD group. Finally, expression data for the four miRNAs among patients are summarized in the heatmap of Figure 2, where color intensities reflect normalized microRNA expression values (log2), allowing visual inspection of the differential patterns mentioned above.

ROC Curve and Diagnostic Performance

ROC curve analysis demonstrated that miR-23a-3p presented the best diagnostic accuracy, with an area under the curve (AUC) of 0.827 (95% CI: 0.705–0.949), sensitivity of 58.3%, and specificity of 100% (Figure 3). The other miRNAs showed moderate performance. miR-140-3p expression revealed an AUC value of 0.713 (95% CI: 0.546–0.879), while miR-486-3p obtained an AUC of 0.707 (95% CI: 0.542–0.872) and miR-151a-3p an AUC of 0.651 (95% CI: 0.464–0.839). DeLong's test for comparison of ROC curves among the selected microRNAs indicated that miR-23a-3p performed significantly better than miR-140-3p (AUC difference = 0.114; p = 0.007) and miR-151a-3p (AUC difference = 0.176; p = 0.008). No significant differences were observed between miR-23a-3p and miR-486-3p (p = 0.255), nor between the other miRNA combinations (p > 0.05 for all comparisons) (Supplementary Table 1). Spearman's correlation analysis revealed strong positive associations between the expression of all miRNA pairs in the ASD group (Supplementary Figure 1). A very strong correlation was observed between miR-23a-3p and miR-151a-3p (ρ = 0.973), followed by the pairs between miR-23a-3p and miR-140-3p (ρ = 0.918) and between miR-151a-3p and miR-140-3p (ρ = 0.894). miR-486-3p showed moderate to strong positive correlations with the other miRNAs, notably the association with miR-23a-3p (ρ = 0.775), miR-151a-3p (ρ = 0.739), and miR-140-3p (ρ = 0.703).

Integration of microRNA Targets with Tissue Expression and Autism Risk Genes

Data available from TargetScanHuman 8.0 for microRNAs hsa-miR-151a-3p, hsa-miR-23a-3p, hsa-miR-486-3p, and hsa-miR-140-3p (isoforms .1 and .2) revealed 18,750 miRNA-target interactions. The hsa-miR-140-3p.1 and hsa-miR-140-3p.2 isoforms were compiled into a single hsa-miR-140-3p identifier, retaining the highest interaction value in the analysis. Accordingly, upon integrating data with the GTEx tissue expression profile, we identified 11.437 unique target genes, of which 861 genes were cataloged in the SFARI database as associated with Autism Spectrum Disorder (ASD). Finally, Gene Ontology (GO) enrichment analysis of Biological Processes (BP) revealed distinct functional signatures for the different microRNAs analyzed in salivary gland tissue (Figure 4).

In detail, in salivary tissue, gene targets of hsa-miR-140-3p and hsa-miR-486-3p showed strong enrichment in processes related to nervous system development, notably "forebrain development" (adjusted p = 3.10e-08, 36 genes; adjusted p = 4.38e-06, 28 genes) and "telencephalon development" (adjusted p = 4.41e-07; adjusted p = 6.64e-06, 22 genes),

respectively. Processes related to social behavior were also associated with targets of hsa-miR-140-3p (adjusted p = 4.41e-07) and hsa-miR-486-3p (adjusted p = 2.43e-05, 10 genes). hsa-miR-23a-3p (208 expressed genes) was primarily associated with dendrite extension (adjusted p = 1.55e-05) and epigenetic regulation of gene expression (adjusted p = 0.0058), while collectively, hsa-miR-23a-3p (adjusted p = 8.08e-03, 14 genes), hsa-miR-486-3p (adjusted p = 4.03e-05, 22 genes), and hsa-miR-151a-3p (adjusted p = 9.66e-03, 12 genes) significantly regulate genes associated with cognition pathways (GO:0050890).

Enrichment analysis of selected microRNA gene targets expressed in the salivary gland revealed significant enrichment for terms related to telencephalon and forebrain development, correlating these targets with embryogenic processes of neural tissues. Accordingly, we investigated whether the gene targets of these microRNAs exhibit distinct expression profiles between prefrontal cortex (Figure 5a) and hypothalamic tissues (Figure 5b). The integrated analysis of all microRNA gene targets demonstrates that, in both the cortex and the hypothalamus, the analyzed microRNAs regulate targets predominantly involved in neurosynaptic, cognitive, and neuronal excitability processes, with highly concordant functional profiles between the two tissues.

In the cortex, the integrated analysis of all microRNAs revealed 772 unique expressed genes, significantly enriched in cognitive processes (adjusted p = 2.99e-20), learning or memory (adjusted p = 1.44e-19), regulation of membrane potential (adjusted p = 6.46e-20), and synapse assembly (adjusted p = 3.49e-18). Additionally, enrichment was observed for glutamatergic synaptic transmission (adjusted p = 3.20e-16) and postsynaptic organization (adjusted p = 1.36e-14). In the hypothalamus, with 763 unique expressed genes, the most significantly enriched processes were also regulation of membrane potential (adjusted p = 1.43e-18), cognition (adjusted p = 1.51e-18), and synapse assembly (adjusted p = 2.37e-17). Also noteworthy were regulation of synaptic plasticity (adjusted p = 1.14e-16), glutamate receptor signaling pathway (adjusted p = 2.00e-14), and action potential (adjusted p = 9.02e-13).

Finally, Pearson correlation between the expression profiles of gene targets of selected microRNAs in different tissues revealed tissue-specific associations in co-expression (Table 3). In the salivary gland, a strong positive correlation was observed between targets of microRNAs hsa-miR-23a-3p and hsa-miR-486-3p (r = 0.77) and between targets of hsa-miR-23a-3p and hsa-miR-140-3p (r = 0.72), while correlations in cortical tissue were moderate

between targets of hsa-miR-23a-3p and hsa-miR-140-3p (r = 0.69). In turn, in the hypothalamus, a very strong correlation was evidenced between targets of microRNAs hsa-miR-23a-3p and hsa-miR-140-3p (r = 0.96), suggesting possible joint co-expression in this tissue. Collectively, these results demonstrate that the correlation between microRNA targets is highly tissue-dependent, with emphasis on the strong association between gene targets of hsa-miR-23a-3p and hsa-miR-140-3p expressed in the hypothalamus.

Table 3. Pearson correlation between expression profiles of microRNA gene targets in different tissues. Correlation matrix showing Pearson correlation coefficients (r) between the expression profiles of predicted target genes (TargetScanHuman 8.0) for microRNAs hsa-miR-23a-3p, hsa-miR-140-3p, hsa-miR-486-3p, and hsa-miR-151a-3p, assessed in three tissues: salivary gland, cortex (prefrontal), and hypothalamus. r values range where values close to 1 indicate strong positive correlation.

miRNAs	Tissue											
	Salivary Gland				Cortex				Hypothalamus			
	miR-23a-3p	miR-140-3p	miR-151a-3p	miR-486-3p	miR-23a-3p	miR-140-3p	miR-151a-3p	miR-486-3p	miR-23a-3p	miR-140-3p	miR-151a-3p	miR-486-3p
miR-23a-3p	1	0.72	0.05	0.77	1	0.69	0.01	0.14	1	0.96	0.04	0.06
miR-140-3p	0.72	1	0.1	0.57	0.69	1	0.23	0.44	0.96	1	0.08	0.07
miR-151a-3p	0.05	0.1	1	0.07	0.01	0.23	1	0	0.04	0.08	1	0.01
miR-486-3p	0.77	0.57	0.07	1	0.14	0.44	0	1	0.06	0.07	0.01	1

Cellular and Molecular Dysregulation of Gene Pathways Associated with Salivary microRNAs

For functional enrichment analyses, we selected a set of 861 predicted target genes from microRNA-mRNA interactions with high confidence (Supplementary Figure 2). Analysis of the predictive performance of microRNAs, assessed by the Contextual Weighted Correlation Score (CWCS) interaction value, revealed substantial variations in prediction capacity among the different microRNAs analyzed. The microRNA hsa-miR-486-3p presented the best mean CWCS (-0.198 ± 0.210), predicting a total of 434 interactions involving 434 distinct genes, indicating a one-to-one prediction relationship between this microRNA and its targets. hsa-miR-140-3p demonstrated the highest number of predicted interactions (n = 576), with a mean CWCS of -0.113 ± 0.114, encompassing 576 target genes. hsa-miR-151a-3p and hsa-miR-23a-3p presented mean CWCS values of -0.092 ± 0.088 and -0.074 ± 0.112, respectively, with 237 and 302 predicted interactions. It is important to note that, although hsa-miR-486-3p presented

the best mean CWCS, all evaluated microRNAs exhibited mean values close to zero, suggesting that the overall predictive strength is modest when considering the average of all interactions. However, the wide variability observed (standard deviations between 0.088 and 0.210) indicates that specific interactions within each microRNA may present substantially stronger predictions, as evidenced by the minimum CWCS values that reached -1.40 for hsa-miR-486-3p and -0.933 for hsa-miR-23a-3p.

In order to explore the biological significance of the predicted target genes with high confidence (more negative CWCS), we performed functional enrichment analyses using the ShinyGO 0.85.1 platform (Figure 6). The target genes of selected microRNAs were represented for Gene Ontology Cellular Component (CC) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. The results in Figure 6a represent the hierarchical clustering tree of significantly enriched GO-CC terms, where pathways with shared genes are grouped, and point size reflects statistical significance (-log10 FDR). Network analyses of significantly enriched GO-CC terms were constructed in Figure 6b. Complementarily, Figure 6c details the KEGG synaptic vesicle cycle pathway, highlighting predicted target genes involved in cellular processes of synapses and synaptic vesicle recycling, which emerged as central pathways in the enrichment analyses.

4. Discussion

This study provides novel evidence of salivary microRNA dysregulation in Brazilian adults with ASD, a population underrepresented in molecular autism research. Most previous studies have focused on pediatric cohorts from high-income countries, limiting the generalizability of findings to adults and populations in low- and middle-income countries, such as Brazil. By investigating salivary microRNA expression in this context, our findings contribute to a more inclusive understanding of ASD biology and demonstrate the feasibility of saliva as a non-invasive diagnostic fluid in resource-limited settings.

Among the four microRNAs analyzed, three (miR-23a-3p, miR-140-3p, and miR-486-3p) were significantly overexpressed in adults with ASD (Table 2). Cellular component enrichment analyses reported the association of predicted genes regulated by these microRNAs functionally implicated in processes central to neurodevelopment. Pathways such as synaptic and glutamatergic postsynaptic mechanisms, dendritic spines and extension, cognition, forebrain and telencephalon development, and social behavior are associated with genes

predicted to be dysregulated in ASD (Figure 5.a–d). The interaction between expressed microRNAs and their predicted ASD-related gene targets favors the clinical investigation of regulatory networks of interest for neurodevelopment. Thus, their elevated expression supports the potential use as accessible and non-invasive biomarkers, as emphasized by [11], who proposed that ideal biomarkers for ASD should be both neurofunctionally relevant and detectable in biofluids such as saliva.

In our results, the salivary microRNA expression profiles observed in adult participants from the ASD group are consistent with previous studies in pediatric populations, suggesting microRNAs as stable biomarkers across developmental stages [21, 22]. A study of salivary microRNA sequencing in children with ASD reported downregulation of miR-23a-3p and other cluster-related microRNAs, along with predictive associations with adaptive behavior scores, particularly in neurodevelopmental domains [11]. In contrast, miR-140-3p showed positive dysregulation, consistent with the pattern observed in adults in the present study. This consistency across studies suggests a stable expression pattern with clinical relevance. Gene targets of microRNAs involved in transcriptional regulation [23], synaptic signaling, and neural plasticity [24,25] reinforce the mechanistic relevance for biomarkers in ASD.

In our findings, we verified the prediction of tissue co-expression among microRNA targets in salivary gland tissues and brain regions—prefrontal cortex and hypothalamus. Biological processes such as regulation of membrane potential, cognition, and synapse organization are attributed to the gene targets of the selected salivary microRNAs in this study (Figures 5–6). The subtle regulation of each microRNA target may illustrate the mechanistic action between molecular factors and neurodevelopment. For instance, miR-23a-3p regulates key synaptic genes such as neurexin 1 (NRXN1) and the scaffold protein SHANK3, which are associated with genes potentially dysregulated in ASD [26]. The expression levels of miR-140-3p further illustrate this complexity. Its robust expression in both the cerebral cortex and peripheral tissues highlights its ability to connect central and systemic mechanisms, further supporting its potential as a biomarker to capture ASD heterogeneity [27]. Furthermore, the elevated expression of miR-486-3p activates the translational repression of AT-rich interaction domain 1B (ARID1B), a gene that has been predicted as a target of this miRNA [28]. This gene has significant function in central nervous system development and maturation processes, as well as in neuronal differentiation, dendritic arborization, and synapse formation [29,30,31,32].

We also evidenced differential microRNA expression between subcategories of the ASD group, when related to the patient's year of diagnosis. Clinical diagnostic patterns regarding autism have undergone etiological changes up to the definition of this neurodevelopmental disorder [15]. Here we report the dysregulation of miR-486-3p, whose interaction with gene targets such as ARID1B may provide irregularities in synaptic development evolution, being correlated with both impaired intelligence and autistic behavior [28,33]. Thus, the salivary microRNA expression profile associated with clinical comorbidities in ASD patients may indicate correlations with the individual's symptomatological presentation, or with conditions relevant to the clinical diagnostic process (Figure 2). Studies on microRNA expression in serum from ASD patients have reported differential expression profiles when associated with comorbidities such as Tourette syndrome [34]. Previous associations between microRNA expression and behavioral performance in pediatric patients, using clinical tools such as the Well-being of Young Children (SWYC), further support this relationship, although the mechanistic pathways related to development remain to be elucidated [23]. Furthermore, no significant alterations in microRNA expression were observed between patient support levels, where multiple therapeutic effects in favor of the patient's neurodevelopment may be associated with significant expression stabilizations.

Our results also indicate elevated expression of miR-23a-3p in patients from the ASD group and co-expression between this microRNA and miR-140-3p and miR-486-3p. Notably, findings related to miR-23a-3p have been associated with the regulatory control of gene targets involved in synaptic growth pathways and inflammatory signaling relevant to ASD. Elevated miR-23a-3p expression levels have been reported in peripheral blood mononuclear cells of children with ASD, along with increased IL-6 expression, a pro-inflammatory cytokine implicated in ASD-related immune dysfunction [22]. As IL-6 can cross the blood-brain barrier and influence cognitive pathways, its correlation with miR-23a-3p supports a neuroimmune mechanism in ASD pathology predicted in regulatory networks in ASD [35]. Thus, the observed overexpression in miR-23a-3p could therefore reflect molecular dysfunctions encompassing both the immune and synaptic systems. Consequently, suppression of target gene expression would lead to alterations in parts of the individual's neuronal development, which may be accentuated over time and with the patient's established treatment. Additionally, expression analyses of miRNAs found in serum and lymphoblastoid cell lines from autistic individuals also indicate a positively regulated miR-486 expression profile in both cases, highlighting the microRNA as a promising tool for molecular screening [36].

Regarding biological processes in the human brain, studies also report that miR-140-3p is more expressed in the human cerebral cortex and in various human blood cell components [37,38]. Target genes of miR-140-3p, such as NRIP1 and CD38, presented in the study by [39], play a crucial role in hormonal regulation, immunological development, and cognition [39]. For example, the CD38 enzyme is a multifunctional molecule present in hematological samples, involved in cell signaling and homeostasis, and in immune cell activation. However, CD38 expression is also elevated in the brain, particularly in the hypothalamus [40]. Cooperatively, this protein plays a crucial role in brain development and function, particularly in emotional and cognitive processes [41,42]. Thus, the correlation between microRNAs and gene targets may interconnect in the construction of regulatory networks aimed at screening for neurodevelopmental conditions, such as pathways related to the development of brain structures in the embryonic phase, including the forebrain and telencephalon. Although in the present study no significant difference in salivary miR-151a-3p was recorded between the control and ASD groups, a high-regulation variance of this microRNA is perceived in the control group. We therefore suggest that for a complementary view of salivary microRNA quantification results, analyses regarding population and sample size of the analyzed groups, as well as quantitative data for identification of the patient's clinical profile, are pertinent to the correlation between microRNAs and gene targets. Recent findings identified elevated levels of miR-151a-3p in the saliva of children with ASD and those at risk for developmental delays, suggesting its potential stable expression level across developmental stages [21].

In this study, 163 predicted target genes regulated by the selected microRNAs were identified, with more negative CWCS values (< -0.3) indicating higher-confidence miRNA-mRNA interactions. These targets help clarify how salivary microRNA expression plays a role in the regulation of genes related to neuronal signaling and neurodevelopment. In chemical synapses, vesicle-membrane fusion events are mediated by the interaction of SNARE complex proteins during the synaptic vesicle cycle (Figure 6) [43]. Accordingly, a distinct class of neurological disorders related to dysregulation of Synaptosomal-associated protein 25 kDa (SNAP-25) and vesicle-associated membrane protein 2 (VAMP2) are associated with developmental delay, motor problems, and ASD [44]. Deficits in neurotransmitter release via neuronal SNARE vesicles would thus impair the synchronization of vesicle-membrane fusion during action potential arrival between neurons [44,45]. In this sense, interactions between regulatory microRNAs of VAMP2 and SNAP-25, when in scenarios of elevated expression, suggest sensitized and compromised molecular mechanisms relevant to the distinct profile of

synaptic molecular functions between neurodivergent groups. Thus, analyses between co-expression of microRNAs and predicted gene targets may clarify the interactivity between molecules and cellular functionality, enabling regulatory panel tools as promising for molecular screening and ASD diagnosis.

Growing evidence suggests that neural cell adhesion genes are also dysregulated in the central and peripheral nervous systems in ASD, playing a role in the remodeling of neuronal migration and neurite outgrowth in the brain [46]. For example, heterozygous deletions and duplications of the CHL1 gene have been reported in findings by [46], where the emergence of phenotypic symptoms of developmental delay in an ASD patient favors neuromolecular tracking processes. Genes related to development and cell growth are also implicated in conditions that compromise neurological development, such as Lessel-Kreienkamp syndrome [47,48]. Cell growth genes are also putative targets for microRNAs [49], whose dysregulation may impact developmental pathways and intracellular signaling. Finally, genes related to signaling pathways of synaptic differentiation processes and neuronal plasticity may be associated with language development disorders [50]. Thus, the expression and activity of genes related to neurological adhesion, growth, and developmental pathways may be modulated by microRNAs, with distinct interaction precision indices, creating a complex and multi-layered regulatory network.

The findings of the present study demonstrate, in an integrated manner, that salivary microRNA dysregulation in Brazilian adults with ASD reflects molecular alterations in central neurobiological pathways, encompassing from embryonic forebrain and telencephalon developmental processes to fine synaptic mechanisms, such as the synaptic vesicle cycle, as well as cognitive and social behavior functions. The significant overexpression of miR-23a-3p, miR-140-3p, and miR-486-3p in ASD patients, associated with the regulation of key genes, highlights the connection between salivary expression and central pathological mechanisms, including neuroimmune dysfunction, synaptic plasticity, and neuronal differentiation. Additionally, the prediction of tissue gene co-expression between salivary gland and prefrontal cortex and hypothalamic tissues stands out, particularly the very strong correlation between miR-23a-3p and miR-140-3p targets in the hypothalamus, validating saliva as a biofluid representative of neurobiological processes. Although miR-151a-3p did not reach statistical significance, its upward trend in the ASD group and its very strong correlation with miR-23a-3p suggest that, in combined panels, it may contribute to diagnostic accuracy, especially considering its stability across development. Finally, the dysregulation of miR-486-3p

associated with the year of diagnosis and its interaction with genes involved in cognitive processes and synaptic vesicle functioning reinforce the hypothesis that alterations in synaptic evolution are correlated with cognitive and behavioral phenotypes of the autism spectrum.

In summary, this pioneering study in the Brazilian adult population with ASD establishes that salivary microRNAs miR-23a-3p, miR-140-3p, and miR-486-3p are promising non-invasive biomarkers, with significantly elevated expression and functional relevance in neurodevelopmental, synaptic plasticity, and cognitive pathways, with miR-23a-3p standing out with the best diagnostic accuracy (AUC=0.827; specificity=100%). The integration between salivary expression, gene target prediction, and tissue co-expression with strategic brain regions reinforces the clinical potential of these microRNAs as molecular tools for screening and diagnostic support of ASD, particularly in resource-limited settings, although expanded population studies are necessary to validate regulatory panels that capture the clinical and etiological heterogeneity of the disorder.

5. Limitations and Future research

Although limited by a small sample size, this study identified significant differential expression of salivary microRNAs specifically miR-23a-3p, miR-140-3p, and miR-486-3p in Brazilian adults with autism spectrum disorder compared to neurotypical controls, offering preliminary support for the utility of salivary microRNAs as non-invasive biomarkers for ASD. While these findings underscore the molecular distinctiveness of individuals with ASD, the study's focus on biological markers to the exclusion of detailed behavioral or communicative assessments restricts the ability to correlate molecular profiles with functional outcomes. This limitation is particularly relevant given prior evidence suggesting that microRNA dysregulation may be associated with adaptive behavior and neurodevelopmental functioning. In summary, the involvement of the studied miRNAs in competitive regulatory networks with other non-coding RNAs reinforces their role in the etiology of ASD and solidifies them as promising candidates in the search for biomarkers for the disorder, warranting more detailed future investigation.

It is important to note that the limited sample size (n = 45) may not only constrain the generalizability of the findings but also affect the precision of the statistical estimates themselves. Small samples are known to reduce the stability of ROC-derived metrics, potentially inflating or underestimating AUC values and increasing overfitting risk. For this reason, the diagnostic performance reported here should be interpreted as preliminary. This

work was designed as a pilot approach to support future, larger-scale studies in Brazilian populations. Furthermore, this study was conducted exclusively in adults. While this design provides insight into a more stable neurodevelopmental phase, it limits the direct comparability of our miRNA expression profiles to those of children or adolescents with ASD, in whom developmental dynamics may differentially influence miRNA expression. Future longitudinal studies tracking individuals from childhood into adulthood are needed to define age-specific and trajectory-based biomarker profiles.

Furthermore, as one of the first studies to examine salivary microRNA expression in a Brazilian adult ASD, it contributes essential data from a historically underrepresented population in autism epigenetics research. These initial findings highlight the importance of expanding the geographic and demographic reach of molecular ASD studies, particularly in low- and middle-income countries where population-specific genetic and environmental influences may yield distinct biomarker signatures.

To this end, future research should prioritize larger, multicenter investigations that integrate molecular data with comprehensive behavioral, cognitive, and clinical phenotyping to clarify the functional implications of microRNA dysregulation and to establish cross-population replicability. Collectively, such efforts are critical for validating the translational potential of salivary microRNAs and for moving toward precision diagnostics and stratified interventions in ASD.

6. Declarations

Ethics Approval and Consent to Participate

This study was approved by the Institutional Ethics Committee of Dom Bosco Catholic University (CAAE No. 56241522.9.0000.5162). All participants were adults (over 18 years of age) and provided written informed consent by signing the Free and Informed Consent Form (FICF). For neurodivergent participants, the FICF was signed by their legal guardians. The study was conducted in strict accordance with the ethical standards of Brazilian National Health Council Resolution No. 466/2012 and with the 1964 Declaration of Helsinki and its later amendments.

Consent For Publication

All participants (or their legal guardians) expressly consented to the publication of the study data and analyses in an anonymized form, as stated in the signed FICF.

Research Data Availability Statement

Supplementary Material 1 consists of a data table containing information on statistical results and Gene Ontology enrichment analyses performed in RStudio. Sociodemographic and amplification data were excluded from the supplementary material due to the sensitivity of the information regarding the confidentiality of volunteers in the ASD and control groups.

Authorship Contribution (CRediT)

M.Sc. Marcos Fernandes Morales (Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Software; Supervision; Validation; Visualization; Writing - original Draft Preparation; Writing - Review & Editing); Samira Naama Souza Silva (Data curation; Formal analysis; Methodology; Software); Yago Henrique Xavier Dantas (Methodology); M.Sc. Ana Carolina Delmondes de Freitas (Investigation; Methodology); Dra. Julia Cunha Gonzales (Data curation; Investigation; Methodology; Project Administration); M.Sc. Willy Rilston Souza de Jesus (Investigation; Methodology); Rômulo Diniz de Arruda (Formal analysis; Methodology; Software); Nataly Emanuelle Ament de Souza (Methodology); Manoella Tomasine Schmidt (Methodology); Dra. Carina Elisei de Oliveira (Project Administration; Supervision; Writing - Review & Editing); Dra. Alinne Pereira de Castro (Conceptualization; Funding Administration; Resources; Supervision; Writing - original Draft Preparation; Writing - Review & Editing).

Declaration Of Generative AI and AI-assisted Technologies in the Manuscript Preparation Process

During the preparation of this work, the author(s) used DeepSeek (deepseek.com) for script structuring for data analysis, code troubleshooting and optimization in bioinformatics, language translation and grammatical refinement of the manuscript text.. The author(s) reviewed and edited the output as needed and take full responsibility for the content of the published article.

Competing Interests

The authors declare no competing interests.

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