Increased Serum Levels of miR-125b and miR-132 in Fragile X Syndrome
A Preliminary Study

Rowena Rubim Couto, MD, Francyne Kubaski, PhD, Marina Siebert, PhD, Têmis Maria Félix, PhD, Ana Carolina Brusius-Facchin, PhD, and Sandra Leistner-Segal, PhD

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Abstract

Background and Objectives
Fragile X syndrome (FXS) is a neurodevelopmental disorder, identified as the most common cause of hereditary intellectual disability and monogenic cause of autism spectrum disorders (ASDs), caused by the loss of fragile X mental retardation protein (FMRP). FMRP is an RNA-binding protein, a regulator of translation that plays an important role in neurodevelopment, and its loss causes cognitive and behavioral deficits. MicroRNAs (miRNAs) are small molecules that regulate gene expression in diverse biological processes. Previous studies found that the interaction of FMRP with miR-125b and miR-132 regulates the maturation and synaptic plasticity in animal models and miRNA dysregulation plays a role in the pathophysiology of FXS. The present study aimed to analyze the expression of miR-125b-5p and miR-132-3p in the serum of patients with FXS.

Methods
The expressions of circulating miRNAs were studied in the serum of 10 patients with FXS and 20 controls using the real-time quantitative retrotranscribed method analyzed by relative quantification. Receiver operating characteristic (ROC) curves and the area under the ROC curve (AUC) were generated to assess the diagnostic values of the miRNAs.

Results
We found that both miR-125b and miR-132 were increased in the serum of patients with FXS compared with controls and likely involved with FMRP loss. The AUC (95% confidence interval) of miR-125b and miR-132 was 0.94 (0.86–1.0) and 0.89 (0.77–1.0), respectively. Databases allowed for the identification of possible target genes for miR-125b and miR-132, whose products play an important role in the homeostasis of the nervous system.

Discussion
Our results indicate that serum miR-125b and miR-132 may serve as potential biomarkers for FXS. The increased expression of circulating miR-125b and miR-132 seems to be associated with the genotype of FXS. Predicted gene targets of the differentially regulated miRNAs are involved in cognitive performance and ASD phenotype.

Classification of Evidence
This study provides Class III evidence that miR-125b and miR-132 distinguish men with FXS from normal controls.

From the Medical Genetics Service (R.R.C., F.K., M.S., T.M.F., A.C.B.-F., S.L.-S.), Hospital de Clínicas de Porto Alegre-HCPA; Postgraduate Program in Medicine: Child and Adolescent Health (R.R.C., T.M.F., S.L.-S.), UFRGS; and Postgraduate Program in Genetics and Molecular Biology (F.K.), PPGMB, UFRGS, Porto Alegre, RS, Brazil.

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MicroRNAs (miRNAs) are short noncoding RNAs involved in posttranscriptional gene regulation by promoting messenger RNA (mRNA) silencing and influencing protein translation. Several studies have provided evidence that miRNAs are involved in the pathogenesis of different disorders, and disease-specific miRNA profiles have been identified being isolated from bodily fluids (circulating miRNAs), including those selectively expressed in the brain. miRNAs play a role during neurodevelopment to regulate essential biological processes in the functioning of the CNS. The deregulation expression of miRNAs has been found to play a major role in the pathogenesis of neurodisorders. Because of the stability and easy accessibility of circulating miRNAs, their use as diagnostic and prognostic biomarkers for patient stratification is highlighted and the efficacy of targeted treatments for neurodisorders, such as fragile X syndrome (FXS), is increased.

FXS is the most common cause of inherited intellectual disability (ID) and the most prevalent monogenic cause of autism spectrum disorders (ASDs). FXS has an estimated incidence of 1 in 4,000 men and 1 in 8,000 women without known incidence in Brazil. This neurodevelopmental disorder is characterized by a broad spectrum of behaviors, such as delayed neuropsychomotor development (ADNPM), anxiety, aggression, self-injury, attention deficit disorder, social withdrawal, and physical comorbidities, such as facial dysmorphisms, macroorchidism, otitis, and seizures, resulting in a large phenotypic heterogeneity across the FXS population. The syndrome is caused by the loss of fragile X mental retardation protein (FMRP), a consequence of the full mutation (FM), more than 200 cytosine-guanine-guanine (CGG) repeats in the 5’ untranslated region of the fragile X mental retardation 1 (FMR1) gene (OMIM# 309550) that leads to hypermethylation of the promoter region, and consequently the absence of the FMRP. FXS mosaicism has been described as the coexistence of the FM and the premutation (CGG repeats between 55 and 200), and the clinical manifestations of FXS mosaicism may vary according to the presence of different methylation levels of the FM allele leading to differential FMRP expression within tissues.

FMRP is a RNA-binding protein highly expressed in the CNS with essential functions for normal development and maintenance of neuronal synaptic function and plasticity through the role as a regulator of translation of neuronal mRNAs in response to synaptic activity. Reduced expression of FMRP leads to abnormalities in neurodevelopmental spines and disturbed neuronal processes, observed in FXS. The RNA binding capacity of FMRP is central to its molecular function, it has 2 K homology domains and 1 arginine-glycine-glycine box, also both nuclear localization and exportation signals for transport target RNA between the nucleus and cytoplasm. FMRP is associated with polyribosomes to form ribonucleoprotein complexes that regulate translation of certain proteins involved in neuronal development and plasticity, and FMRP functions as a translation repressor at synopsis due to its binding to miRNAs and interactions with proteins, including Argonaute (Aggo2), incorporated into a multiprotein complex called an RNA-induced silencing complex (RISC), resulting in the regulation of synaptic structure and plasticity.

Studies have provided evidence of miRNA involvement in the pathogenesis of FXS by identifying and isolating several r(CGG)-derived miRNAs, as miR-125b and miR-132, required for maintaining neuronal connectivity and synaptic plasticity, in the zebrafish FXS model. Furthermore, microarray analyses of miRNAs associated with FMRP in the mouse brain identified miR-125b and miR-132 and the dysregulation of both miRNA-mediated protein translation resulting in early neural development and synaptic physiology. A recent study showed that FMRP binds with some miRNAs, specifically miR-125-family, in regions outside of the seed sequence to modulate the RISC complex through specific interaction with Ago2 protein. In addition, a recent miRNA profiling performed in the urine of boys with FXS has identified an increase of miR-125a and its potential as an FXS biomarker. In this study, these miRNAs were chosen and analyzed in the serum samples of patients with FXS (n = 10) compared with healthy controls (n = 20) with the aim of investigating whether FXS pathogenesis is associated with miR-125b-5p and miR-132-3p and whether those could be used as diagnostic and prognostic biomarkers of patients with FXS.

**Methods**

**Clinical Samples**

Samples were collected from patients with FXS from the Medical Genetics Service at Hospital de Clínicas de Porto Alegre (HCPA) and from the Clinical Samples of Porto Alegre (HMDD); one hundred healthy controls (n = 100) were also investigated. The analyses were performed in the Genomics Laboratory of the Medical Genetics Service at HCPA.

**Methods**

**Clinical Samples**

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The reverse transcription into complementary DNA (cDNA) was performed using the StepOne (Thermo Fisher Scientific) and the TaqMan Advanced miRNA cDNA Synthesis Kit (Cat. No. A28007), according to the manufacturer’s instructions. Quantitative real-time PCR was performed using the Applied Biosystems QuantStudio 3 PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.), and all miR-specific primers and universal adaptor PCR miRNA primers were purchased from commercial TaqMan miRNA assays (Life Technologies; miR-24-3p [ID 477992_mir], miR-132-3p [477900_mir], and miR-125-5p [477885_mir]). The TaqMan Fast Advanced Master Mix (Cat. No. 4444605) was used for the qPCR. All the reactions were performed in duplicates, the reaction mixtures were incubated in a 96-well plate at 95°C for 20 seconds, and then 40 cycles of reaction were performed at 95°C for 1 second and at 60°C for 20 seconds. Fluorescent signals were measured during the extension phase.

The relative expression levels were calculated using the comparative Ct method with mean values of controls set as a calibrator. The Ct values of miRNAs were standardized and calculated using the 2^−ΔΔCt method for the fold change between patients with FXS and controls. For normalization, miR-24-3p was used as an endogenous control for further statistical analysis.

**Table 1** Mean and Interval Ranges of Circulating miRNA Expression Levels in FXS and Healthy Controls

<table>
<thead>
<tr>
<th>miRNAs</th>
<th>FXS (n = 10)</th>
<th>HC (n = 20)</th>
<th>p Value</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>mir-125b-5p</td>
<td>2.86 (1.46–6.66)</td>
<td>1.12 (0.34–2.392)</td>
<td>&lt;0.001</td>
<td>2.55</td>
</tr>
<tr>
<td>mir-132-3p</td>
<td>2.17 (1.18–3.49)</td>
<td>1.09 (0.37–2.03)</td>
<td>&lt;0.001</td>
<td>2.0</td>
</tr>
</tbody>
</table>

Abbreviations: FXS = fragile X syndrome; HC = healthy control; miRNA = microRNA.

**Statistical Analysis**

All analyses were performed using R software, version 4.0.5 (R Core Team 2020, R-project.org/). As data were not normally distributed, nonparametric statistical tests were used. miRNA expression levels between the patients and controls were analyzed using a 2-sided Mann-Whitney test. Differences in the miRNA expressions between the groups were studied using the same test to evaluate the association between miRNA expressions and clinical parameters (genotypes, age, ID, ASD, comorbidities, and behavioral alterations). Receiver operating characteristic (ROC) curve analysis was constructed, and the area under the ROC curve (AUC) was generated to assess its diagnostic values for evaluating the diagnostic accuracy of circulating miRNAs. p < 0.05 was considered statistically significant.

**Pathway Analysis and Target Genes of Investigated miRNAs**

The mirPath database, which uses experimentally validated miRNA interactions derived from DIANA Tools mirPath v.3 (microrna.gr/mirPathv3), was used to investigate in which pathways the miRNAs of this study are already determined to be involved. Using the Human MicroRNA Disease Database (HMDD V3.0) (cuilab.cn/hmdd), we searched for interaction targets involved in human diseases. We also used the R platform, multiMiR package (R Core Team, R-project.org/), and we searched for interaction targets of miR-125b-5p and...
miR-132-3p involving neurodevelopmental diseases. Targets with experimental validation were chosen through miRwalk (mirwalk.umm.uni-heidelberg.de/) and DIANA Tools TarBase v.8 (microrna.gr/tarbase).

**Data Availability**
The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available as the data contain information that could compromise the privacy of research participants.

**Results**

**Circulating miRNA Expression in the Serum of Patients With FXS and Healthy Controls**

We analyzed the expression of circulating miR-125b-5p and miR-132-3p in the serum obtained from 10 patients with FXS and 20 healthy controls (HCs). miR-125b-5p and miR-132-3p were identified by qRT-PCR. Analysis of the miRNA expressions between the groups revealed overexpression in patients with FXS of miR-125b-5p and miR-132-3p (fold change, FC > 1.5; \( p < 0.05 \)) (Table 1) in comparison to controls. miR-125b-5p (\( p = 1.79 \times 10^{-5} \)) (Figure 1A) and miR-132-3p (\( p = 2.58 \times 10^{-4} \)) (Figure 1B) were increased in the FXS serum.

ROC curves were constructed, and the AUC was generated to assess the diagnostic values of both selected miRNAs. As shown in Figure 2, ROC analysis revealed that the AUC of miR-125b-5p and miR-132-3p was strongly increased (Figure 3A and Figure 3B). The FM group showed significant overexpression of miR-125b-5p (\( p = 1.5 \times 10^{-4} \)) and miR-132-3p (\( p = 7.15 \times 10^{-4} \)) in comparison with controls, and mosaic patients showed significant overexpression of miR-125b-5p (\( p = 4.25 \times 10^{-3} \)) and miR-132-3p (\( p = 2.36 \times 10^{-2} \)) in comparison with controls. The expression of miR-125b-5p and miR-132-3p in the FM genotype was upregulated in comparison with mosaic patients, but statistical significance was not observed (miR-125b-5p, \( p = 0.0952 \); miR-132-3p, \( p = 0.1508 \)).

**Pathway and Target Gene Analysis of Investigated miRNAs**

Using the miRSystem tool, more than 600 pathways were found. The miRNAs were involved separately, such as the mitogen-activated protein kinase (MAPK) signaling pathway, pathways in cancer, neurotrophic signaling, and immune adaptive system.\(^{14}\) The pathway ranking summary revealed that both miRNAs were involved in 13 pathways: signaling by transforming growth factor beta (TGF-\( \beta \)) signaling pathway, regulation of the actin cytoskeleton, bacterial invasion of epithelial cells,
hippocampal signaling pathways, lysine degradation, proteoglycans in cancer, other types of O-glycan biosynthesis, adherens junction, cell cycle, arrhythmogenic right ventricular cardiomyopathy, biosynthesis of unsaturated fatty acids, fatty acid biosynthesis, and fatty acid metabolism. Figure 4 represents a heatmap of union pathways of both miRNAs derived from experimentally validated data using miRPath v.3, DIANA Tools Database.

There are 925 predicted targets for miR-125b-5p and 673 predicted targets for miR-132-3p in miRDB. Using the HMDD, we found that for both miRNAs investigated, there are at least 4 confirmed target genes in humans. Using the multimIR platform, it was possible to generate a list of target genes for miR-125b-5p and miR-132-3p that are related to neurodevelopmental diseases. We identified 4 possible target genes by miR-125b-5p including insulin-like growth factor 1 receptor (IGF1R), tumor protein p53 (TP53), glutamate ionotropic receptor NMDA type subunit 2A (GRIN2A), and single-minded 1 (SIM1) and 5 genes targeted by miR-132-3p, including SRY-box 5 (SOX5), SRY-box 6 (SOX6), mitogen-activated protein kinase 1 (MAPK1), brain-derived neurotrophic factor (BDNF), and phosphatase and tensin homolog (PTEN) (Table 4).

Discussion

The search for noninvasive biomarkers for early diagnosis and disease prognosis is currently one of the most rapidly growing areas in neurodevelopmental research. In the serum of patients with FXS, circulating miRNAs could be used as prognostic neuropathologic biomarkers. Recently, the blood serum was found to contain circulating miRNAs, which are stable, reproducible, and have already been proposed as novel non-invasive biomarkers for the diagnosis and prognosis of many neurodisorders. In this study, FXS-specific changes in serum miRNAs were identified. The levels of miR-125b and miR-132 were found increased in the FXS serum, compared with the serum samples of HCs (Figure 1), suggesting an important role of these miRNAs in the pathophysiology of FXS.

Previous studies have demonstrated that miR-125b and miR-132 are associated with FMRP in the mouse brain, and this association is necessary to affect dendritic spine morphology. In addition, miR-125b and miR-132 regulation is affected in the absence of the FMRP, which is in agreement with our results. Through the phosphorylation state of FMRP, influenced by mGluR and when phosphorylated, FMRP is associated with miR-125b and miR-132 to form the RISC complex, which, when induced by those miRNAs, regulates postsynaptic density protein 95 (PSD-95) translation, which is critical for the synaptic function. In the brain of the FMR1 KO mouse, the levels of miR-125 and miR-132 were reduced, explaining the inability of miR-125– and miR-132–guided RISC complex to regulate PSD-95 mRNA, resulting in dendritic abnormalities. PSD-95 is a postsynaptic scaffolding protein that modulates the synaptic formation, maintenance, and localization by forming related signals with the NMDA receptor, which plays an important role in synaptic transmission. The mGluR5 receptor binds to the NMDA receptor through PSD-95, which directly affects synaptic plasticity. It was demonstrated that miR-125b targets the NR2A subunit of the NDMAR that influences synaptic plasticity and memory consolidation and the association between miR-125-family and mGluR5 signaling to the inhibitory complex on PSD-95 mRNA, miR-132 is also linked to NMDA receptors. Pharmacologic studies showed that miR-132 induction requires the NMDA receptor activation, and recently, miR-132 was shown to participate in regulating the expression of PSD-95 through target genes. Possibly, miR-125b and miR-132 interact with FMRP by other sequential signaling pathways, such as mGluR and NMDA receptors signaling, to mediate the PSD-95 translation. Thus, any destabilization of these molecules may lead to abnormal synaptic plasticity, but the molecular mechanisms by which this regulation is accomplished are still not clear.

<table>
<thead>
<tr>
<th>Table 2 Receiver Operating Characteristic (ROC) Analysis of miRNAs in Patients With FXS</th>
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<tbody>
<tr>
<td>miRNAs</td>
</tr>
<tr>
<td>-----------------</td>
</tr>
<tr>
<td>miR-125b-5p</td>
</tr>
<tr>
<td>miR-132-3p</td>
</tr>
</tbody>
</table>

Abbreviations: AUC = area under the receiver operating characteristic curve; CI = confidence interval; FXS = fragile X syndrome. p Value, compared with AUC of 0.5.
The phenotype analysis failed to reveal a significant correlation between the miRNA levels, considering that the sample size is a limitation in this study. Studies performed using miRNA have been related to many neuropsychiatric diseases, such as anxiety disorder, ASD, and hyperactivity. In our study, the stratification of the patients with FXS by clinical characteristics only evidenced the phenotypic heterogeneity beyond the disease. Moreover, the severity of the FXS phenotype varies individually and depends on both the dosage and the length of the CGG repeat. The behavioral phenotype that includes a majority of attention disorders such as ID, hyperactivity, mood instability, aggressivity, and abnormalities in sensory stimuli as in ADNPM was found in most of our patients. Although epilepsy is associated with FXS in about 15%–20% of males with FXS, the patients included in this study did not present this disorder. In addition, miR-132 was found significantly higher in children with attention deficit and hyperactivity, and miR-125b was identified to be upregulated in 20 patients with ASD compared with controls. Whereas those are common characteristics in FXS, any correlation between them would be solely a hypothesis.

A regulation, whereas FM patient samples showed higher serum expression levels of miR-125b and miR-132, which underline the absence of FMRP protein may further directly influence the gene expression of these miRNAs, since mosaic patients express low levels of FMRP. Previously, miR-125b and miR-132 were reported to be severely decreased in the mouse brain due to FMRP knockdown. Recently, miR-125a was increased in the urine of 9 patients with FXS, compared with HCs. The FM genotype patients with higher levels of miR-125b and miR-132 in the serum may present a subgroup whose cellular homeostasis differs from the mosaic genotype subgroup, with lower levels of miR-125b and miR-132. The higher serum miR-125b and miR-132 levels in FM patients could be a consequence of the lack of FMRP interaction and may reflect increased production and/or brain secretion of these miRNAs in corporal fluids, whereas the expression of FMRP could decrease the high serum levels of miR-125b and miR-132. However, it is not possible to make any specific directly correlation between the expression of circulating miRNAs in human fluids and previous findings in mouse brain miRNAs levels. Thus, it becomes necessary to invest in further studies that seek to better understand the deregulation of the levels of these miRNAs observed in FXS. Moreover, these miRNAs are found related to different neuropathologies, which demonstrates their involvement with several pathways’ regulation.

Previously, serum miR-125b was found to be increased in patients with multiple sclerosis and Alzheimer disease, respectively.

### Table 3 Clinical Characteristics of Patients With Different Genotypes and Healthy Controls

<table>
<thead>
<tr>
<th></th>
<th>Full mutation (n = 5)</th>
<th>Mosaic premutation/full mutation (n = 5)</th>
<th>Healthy controls (n = 20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y), mean ± SD (min-max)</td>
<td>15 ± 3.9</td>
<td>17 ± 3.3</td>
<td>21 ± 3.9</td>
</tr>
<tr>
<td>ID, n (%)</td>
<td>5 (100)</td>
<td>3 (60)</td>
<td>—</td>
</tr>
<tr>
<td>ASD, n (%)</td>
<td>1 (20)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>ADNPM, n (%)</td>
<td>2 (40)</td>
<td>4 (80)</td>
<td>—</td>
</tr>
<tr>
<td>Aggressivity, n (%)</td>
<td>—</td>
<td>2 (40)</td>
<td>—</td>
</tr>
<tr>
<td>Hyperactivity, n (%)</td>
<td>4 (80)</td>
<td>2 (40)</td>
<td>—</td>
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<tr>
<td>Hypothyroidism, n (%)</td>
<td>—</td>
<td>1 (20)</td>
<td>—</td>
</tr>
<tr>
<td>Obesity, n (%)</td>
<td>1 (20)</td>
<td>1 (20)</td>
<td>—</td>
</tr>
</tbody>
</table>

Abbreviations: ADNPM = delayed neuropsychomotor development; ASD = autism spectrum disorders; ID = intellectual disability.
associated with severe cognitive decline, and described as a potential biomarker for these neuropathologies. miR-125b transfection into neuronal cells caused hyperphosphorylation of tau, induced oxidative stress, inflammation, and apoptosis, and inhibited cell proliferation, exemplifying how the dysregulation of this miRNA can affect the biochemistry of the brain, contributing to the onset of neuropathologies.\textsuperscript{25} Of interest, a study described the inhibitory effect of miR-125b results in the upregulation of multiple miR-125b target genes, including \textit{IGF1R}.\textsuperscript{26} \textit{IGF1R} is a transmembrane tyrosine kinase receptor essential for neuronal development, has been found to regulate changes in neuronal polarity, and has neuroprotective effects after brain injury.\textsuperscript{27} miR-132 is the most studied miRNA linked to brain function, predominantly observed dysregulated in neuropathologies and regulated in response to neuronal activity. Many studies have shown that miR-132 increased in the blood components of neuropathologies, such as Alzheimer disease,\textsuperscript{28} Parkinson disease,\textsuperscript{29} MS,\textsuperscript{30} and amyotrophic lateral sclerosis,\textsuperscript{31} which is in accordance with the higher levels found in our study, and it highlights its overexpression related to neuropathologic processes and its potential as a biomarker of neuropathologies.

The experimental heatmap showed miR-125b-5p and miR-132-3p connected to cancers and cycle cellular pathways (Figure 4). Both miRNAs are involved with oncogenic aspects demonstrating the potential of biomarkers for the screening of different types of cancer. miR-132-3p could be a novel biomarker for screening of hepatocellular, lung, and nasopharyngeal carcinoma,\textsuperscript{32} while miR-125b-5p could be the same for lung cancer and breast cancer.\textsuperscript{33} The validated target \textit{TP53} gene for miR-125b plays a key role in cell death, DNA repair, and cell proliferation, and the overexpression of miR-125b represses the endogenous level of the P53 protein.\textsuperscript{34} A \textit{TP53} review highlighted a key role for the gene in the regulation of neurons, which highlights the complexity of this target in neuropathologies.\textsuperscript{35} miR-125b-5p and miR-132-3p are related to signaling pathways

\begin{table}[h]
\centering
\caption{List of Validated Target Genes of miR-125b-5p and miR-132-3p According to the Human MicroRNA Disease Database and multimir Package (R Platform), Validated by miRwalk and DIANA Tools TarBase v8}
\begin{tabular}{|l|l|}
\hline
\textbf{miRNA} & \textbf{Target genes} \\
\hline
\textit{miR-125b-5p} & \textit{IGF1R} \\
& \textit{TP53} \\
& \textit{GRIN2A} \\
& \textit{SIM1} \\
\hline
\textit{miR-132-3p} & \textit{SOX5} \\
& \textit{SOX6} \\
& \textit{MAPK1} \\
& \textit{BDNF} \\
& \textit{PTEN} \\
\hline
\end{tabular}
\end{table}
of fatty acid biosynthesis and fatty acid metabolism, and circulating levels of both miRNAs are related to body weight.\textsuperscript{36} Obesity has already been demonstrated to affect gene expression and was associated with cognitive decline and dementia.\textsuperscript{37} Another related pathway between the miR-125b-5p and miR-132-3p is the TGF-β signaling, which play essential roles in every stage of neural development, showing the importance of both miRNAs in the pathways of TGF-β members in the CNS at various developmental stages.\textsuperscript{38} In fact, miRNAs can regulate hundreds of different targets involved in different pathways, and these are just some routes involving both miRNAs.

The experimentally validated gene targets showed that the miRNAs have been connected to genes involved in cognitive performance and ASD (Table 3). MiR-125b is involved with GRIN2A downregulation, and this gene encodes an important subunit of NR2A, a subunit of NMDA receptor.\textsuperscript{11} Variants of GRIN2A have been described as associated with several neurodevelopmental disorders, including epilepsy and ASD.\textsuperscript{39} Another target gene for miR-125b is SIM1, involved with behavioral disorders, ASD, and obesity in humans.\textsuperscript{40,41} miR-132 was found to reduce SOX5 mRNA and protein expression.\textsuperscript{42} Subsequent to this finding, SOX5 had been identified as a novel candidate gene for ASD.\textsuperscript{43} Furthermore, miR-132-3p was found to target SOX6 and downregulated its protein expression.\textsuperscript{44} SOX6 variants have been reported to cause a neurodevelopmental syndrome associated with attention deficit and hyperactivity disorder.\textsuperscript{45} Another gene target and downregulated by miR-132 is MAPK1.\textsuperscript{46} MAPK signaling regulates intracellular functions, and perturbations to MAPK signaling are related to contribute to the pathogenesis of ASD.\textsuperscript{37} Therefore, miR-132 was found to improve the cognitive function of rats by directly inhibiting MAPK1 expression.\textsuperscript{46} BDNF is indirectly targeted by miR-132 through methyl CpG-binding protein 2, a known regulator of neuronal maturation and synapse formation, which triggers the induction of BDNF.\textsuperscript{19} A BDNF meta-analysis study has detailed the effects of gene expression in ASD. Higher peripheral BDNF in ASD is concordant with several neurologic and psychological causes and symptoms of this condition, and the lower levels of BDNF were found in schizophrenia, bipolar disorder, and depression.\textsuperscript{46} Recently, miR-132 overexpression demonstrated their downregulated expression of PTEN.\textsuperscript{49} PTEN protein is involved in the regulation of the cell cycle and is important in synaptic plasticity, neuronal function, and development.\textsuperscript{49} PTEN variants have been associated with ASD phenotypes,\textsuperscript{49} and PTEN-KO mice models showed macrocephaly, loss of neuronal polarity, and behavioral anomalies associated with ASD, such as anxiety, convulsions, and decreased social interest.\textsuperscript{50}

Of interest, TGF-β plays important roles in the maintenance of neuron and spine homeostasis, and there are different studies describing the association of the TGF-β signaling pathway with neuronal development and neurologic disorders.\textsuperscript{51} Recently, the transcript of the type 2 BMP receptor (BMPR2) gene, a member of the TGF-β superfamily, has been identified as a novel target of FMRP.\textsuperscript{52} In the brains and neurons of patients with FXS and Fmr1-KO mice, the amount of BMPR2 protein is increased.\textsuperscript{52} Another pathway involved with both miRNAs and correlated with FXS is fatty acid biosynthesis. Fatty acid biosynthesis was found significantly altered in Fmr1 KO2 mice.\textsuperscript{53} Additional studies are needed to elucidate and better understand the correlation between the aberrant activation of this pathway and the FXS pathogenesis.

The AUC values of miR-125b and miR-132-3p in discrimination of patients with FXS from HCs were 0.94 and 0.89, respectively, which demonstrate the ideal diagnostic value to distinguish patients with FXS from healthy individuals. The increased levels may indicate the severity of FXS in patients, contribute to clinical diagnosis, and further patient prognosis; because FXS still has no prognostic tools, developing them is an urgent requirement because the neurodevelopmental disorder is accompanied by uncertain wide-ranging comorbidities. Although the present study still has some limitations, as the samples recruited in our study are relatively small, our findings elucidate the clinical potential biomarkers of serum miR-125b and miR-132 for patients with FXS, and the genetic miRNA associations have shown that targets of miR-125b and miR-132 can determine the onset of neurodevelopmental disorders and ASD phenotypes. A multicenter collaborative study would be of great value to confirm our findings and to allow correlations to the patient’s phenotype to improve the understanding of the FXS regulation by miRNAs.

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Appendix Authors

<table>
<thead>
<tr>
<th>Name</th>
<th>Location</th>
<th>Contribution</th>
</tr>
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<tbody>
<tr>
<td>Rowena Rubim</td>
<td>Medical Genetics Service, Hospital de Clínicas de Porto Alegre-HCPA, RS, Brazil; Postgraduate Program in Medicine: Child and Adolescent Health, UFRGS, Porto Alegre, RS, Brazil</td>
<td>Major role in the acquisition of data; study concept or design; and analysis or interpretation of data</td>
</tr>
<tr>
<td>Couto, MD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Francyn Kubaski, PhD</td>
<td>Medical Genetics Service, Hospital de Clínicas de Porto Alegre-HCPA, RS, Brazil; Postgraduate Program in Genetics and Molecular Biology, PPGMB, UFRGS, Porto Alegre, RS, Brazil</td>
<td>Drafting/revision of the manuscript for content, including medical writing for content, and major role in the acquisition of data</td>
</tr>
</tbody>
</table>
References


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